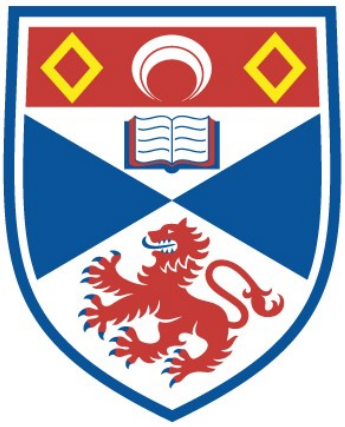
CELLULAR INTERACTIONS IN THE THYMIC MICROENVIRONMENT

Dinesh Andrew Jacob

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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CELLULAR INTERACTIONS IN THE THYMIC MICROENVIRONMENT

A Thesis

Submitted to the Faculty of Science, University of St. Andrews for the degree of Doctor of Philosophy

by

Dinesh Andrew Jacob

Department of Anatomy and Experimental Pathology,
University of St. Andrews

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy, in the University of St. Andrews, entitled "Cellular interactions in the thymic microenvironment" is my own composition and is the result of work done mainly by me during the period of matriculation for the above degree. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Dept. of Anatomy and Experimental Pathology, United College of St. Salvator and St. Leonard, University of St. Andrews under the supervision of Prof. D. Brynmor Thomas and Dr. A.C. Riches.

ACADEMIC RECORD

I graduated from the University of Sussex with a B.Sc. (Hons.) in Biochemistry (Class of degree 2:1) in July 1975. I was awarded an M.Sc. in Immunology from the University of Birmingham in November 1976.

I matriculated as a postgraduate research student of the Dept. of Anatomy and Experimental Pathology, University of St. Andrews in October 1976.

CERTIFICATE

We hereby certify that Dinesh Andrew Jacob has spent nine terms engaged in research work under our supervision, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

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It is my pleasant duty to thank Prof. D. Brynmor Thomas and Dr. A.C. Riches for their helpful guidance and encouragement throughout this study. I would also like to thank Prof. Thomas for making available to me the facilities of his department.

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Finally, I would like to thank my parents for all their sacrifices.

ABSTRACT

The haemopoietic multipotential stem cell differentiates in distinct maturational pathways to generate different populations of blood cells. Differentiation and proliferation within the thymus is a prerequisite of precursors committed to the T-lymphocyte lineage. These differentiative events involve an interaction with the non-lymphoid cells of the thymus.

Monolayers derived from the non-lymphoid stromal elements of the murine thymus were established and maintained in vitro. The cells in culture were characterised on the basis of their morphological and functional features. They were found to be morphologically heterogenous at the light microscopical and ultrastructural level, and largely phagocytic. The possible maturation inducing properties of the cultured thymus cells were investigated. The PHA responsiveness of thymocytes, a feature of more mature lymphocytes was found to be marginally enhanced upon co-culture with these cells or after incubation in their conditioned medium. Similar effects were obtained with monolayers derived from peritoneal exudate cells, but to a lesser extent.

A method of depleting the endogenous lymphoid cells in explants of embryonic thymic tissue in culture was established, enabling the enrichment of the epithelial component. These epithelial thymuses were reconstituted with early undifferentiated haemopoietic cells and more mature lymphoid precursors in vitro. The latter were found to readily repopulate the explants, whereas the less differentiated cells did not.

The lymphocytic cells of the bone marrow were isolated by differential centrifugation. The enriched cells were used as targets to investigate the possible differentiation inducing properties of the thymic monolayer cells, as well as their own capacity to repopulate the thymus in vitro. They were found to be refractory to any such maturational induction, and their thymus-seeding ability was not conclusively resolved. The significance of these findings are discussed with regard to the maturational potential of different haemopoietic cells in the lymphomyeloid tissues.

The cell proliferation kinetics of the thymus during late gestation was investigated. The cell production rate was found to be greatly diminished in pregnancy, during which the spleen was found to sustain an increased extra-medullary erythropoietic activity. The responsiveness to PHA during pregnancy was investigated. The possible causes and consequences of haemopoietic imbalance are discussed, especially with regard to the possibly impaired immune competence of the pregnant animal.

CONTENTS

GENERAL INTRODUCTION

In ancient Greece Theophrastus applied the name "thymus" to the aromatic herb, now commonly known as thyme. The mammalian organ owes its name to the Roman physician Galen who compared its appearance to the leaf of the thyme plant.

Although early investigators speculated on its embryological origins at the end of the last century, an understanding of its microscopical structure was not apparent until the work of the Swedish anatomist August Hammar in the early 1900's. The histogenesis of the organ has been the subject of controversy for nearly a century.

Although the thymus has been recognised as a lymphoid organ for a considerable time, it was not until the classic extirpation and replacement experiments of Jacques Miller in the early 1960s that its role in the acquisition of immunocompetence was established. The vastly increased knowledge of the physiology of the thymus since then has parallelled that of Immunology itself. It was soon realised that the thymus, together with the avian bursa of fabricius or its mammalian equivalent, generate distinct lineages of lymphoid cells, capable of effecting the cellular and humoral components of the immune response respectively.

An endocrine role for the thymus, though long suspected, has more recently been established. Hormonal interactions with other endocrine glands especially the anterior pituitary and adrenal cortex are recognised, and putative thymic hormones have been shown to be similar in their molecular mode of action to other more well defined polypeptide hormones.

The study of most physiological systems have been greatly facilitated by the application of in vitro techniques, they have been successfully applied to the investigation of the thymic micro-environment. They vary from the in vitro culture of cellular monolayers on plastic surfaces to the growth of thymic rudiments in the novel and nutritive environment of chick chorioallantoic membranes.

The target cell for the thymic humoral and microenvironmental effects have been difficult to identify, as no clear morphological differences are visible between the early stages of differentiating blood cells. The cellular candidate for differentiation to the T lineage could be the multipotential haemopoietic stem cell or one of the cellular populations derived from it. The haemopoietic system is unique among cellular differentiation systems in that its stem cell population is capable of self-renewal as well as a source of precursors for specialised blood cells.

Any study of individual cellular compartments within any one system may be further complicated by stem cells common to others, as in the possible relationship between the murine monocyte and osteoclast precursors.

The early investigators of the thymus observed that the organ exhibited an unusual age-related atrophy. More recent studies indicated that the organ was a site of both intense cellular proliferation and cell death, a fact which has led to interesting speculations about its role in the induction of tolerance to self-antigens; or more simply, its function as the finishing school in the education of T lymphocytes.

1. DEVELOPMENT OF THE THYMUS

The mammalian thymus is a lympho-epithelial organ located in the superior anterior mediastinum in most species. The literature on the development and histogenesis of the organ is reviewed by Metcalf and Moore (1971). Embryologically, it is derived as outgrowths of a pair of epithelial chords from the branchial ectoderm of the dorsal third and fourth pharyngeal pouches (Verdun, 1898). These epithelial outgrowths proliferate and branch out in all directions, each joining up to form a single bilobed organ. The epithelium continues to grow into the adjacent tissue, becoming surrounded by a layer of mesenchyme. The growth and branching of these cells gives rise to the medullas of the lobules which make up each lobe in most species. Some cells become arranged in groups to form the Hassall's corpuscles, while most are more loosely packed, joined together by desmosomes. The thymus is the first lymphoid primordium to develop in mammalian embryos. During gestation, lymphoid cells are seen to infiltrate the interstices between the epithelial cells and rapidly become the predominant cells in the organ. The origin of these cells has been the subject of much controversy and speculation until relatively recently. Kollicker (1879) proposed that thymic lymphocytes were derived by direct transformation of the epithelial anlage. His (1885) considered them to have formed from the mesenchyme by invasion of the epithelial anlage. Hammar (1905) postulated that the early thymic primordium was invaded by connective tissue lymphocytes. Early <u>in vitro</u> studies of thymic development failed to demonstrate lymphopoiesis (Emmart, 1936);

later studies using more sophisticated tissue culture methods have resolved conflicting theories on the origin of thymic lymphocytes. Auerbach (1960) obtained growth and lobulation in 12 day embryonic thymic rudiments cultured in plasma clots, but the anlage remained epithelial, without lymphoid development. When mesenchymal and epithelial tissue were separated by trypsinisation, both failed to grow and differentiate; but when recombined, growth and lobulation occurred. This inductive effect of the mesenchyme could also be exerted through a cell-impermeable diffusion membrane. Ball and Auerbach (1960) later demonstrated lymphopoiesis in embryonic thymus cultures within the epithelial compartment, in the presence of mesenchymal tissue. Early transplantation studies by Auerbach (1960 and 1961) showed that normal lymphoid development was obtained in the mouse thymic rudiment when grafted to the anterior chamber of the eye, if both epithelial and mesenchymal components were present. Combinations of non-lymphoid mouse thymic epithelium and chick mesenchyme were also effective. He concluded that epithelium generated the lymphocytes and mesenchyme gave rise to the connective tissue and stroma. Similar observations were noted when 12 day mouse thymus was grafted onto the chick chorioallantoic membrane, the lymphocytes formed were all derived from mouse tissue (Moore and Owen, 1967). Auerbach's findings supported the transformation theory of the epithelial origin of thymic lymphocytes, providing the mouse thymic anlage at 12 days of gestation was devoid of lymphoid cells. However, a number of investigators have demonstrated the presence of large,

basophilic cells with prominent nucleoli in the embryonic thymus at 11 days of gestation, which are morphologically distinct from the epithelium (Smith, 1965; Moore and Owen, 1967; Owen and Ritter, 1969). The use of chromosome markers enabled a more conclusive study of lymphopoiesis in the thymus. Moore and Owen (1967) utilised a sex-chromosome marker system in pairs of male and female chick embryos parabiosed by a vascular union of chorioallantoic or yolk-sac blood vessels. After 4-5 days of incubation post-parabiosis, a vascular yolk-sac anastomosis was established, this being prior to the onset of thymic lymphopoiesis after 7 days. Thymic chimaerism was seen in these embryos, suggesting that blood-borne lymphoid precurors were entering the thymic anlage. These findings gave rise to the haematogenous theory of the origin of thymic lymphocytes. Further studies on the entry of precursor cells to the thymus involved culturing embryonic mouse and chick thymic rudiments of different ages in cell-impermeable diffusion chambers on the chick chorioallantois. The system enabled their development to be studied in isolation without further influx of cells, 10 Day mouse and 7 day chick thymic rudiments failed to become lymphoid; whereas in older rudiments, lymphoid development proceeded (Owen and Ritter, 1969). The origin of these immigrant stem cells was investigated by studies on the ability of various cells to repopulate the irradiated thymus. Such cells have been found in avian embryonic blood, spleen, marrow and yolk-sac (Moore and Owen, 1967), in mammalian yolk-sac (Moore and Metcalf, 1970) and in foetal liver (Taylor, 1964). When the blood-borne cells first arrive in the thymic rudiment, the only other tissues containing

haemopoietic stem cells are the yolk-sac and foetal liver in the mouse and yolk-sac and spleen in chickens. Of these, the mouse foetal liver and avian spleen are considered to be rudimentary themselves at this stage; therefore the pre-thymic lymphoid precursors are thought to originate from the yolk-sac. The large, basophilic cells seen in the thymic rudiment were presumed to be the lymphoid precursors, as its appearance in the thymus correlated with the onset of thymic lymphopoiesis. More conclusive proof was obtained when tritiated thymidine (^{3}H TdR) labelled embryonic haemopoietic cells were injected intravenously into developing embryos. Labelled cells were seen in the 8-9 day chick embryonic thymus, first in the blood vessels and connective tissue surrounding the thymic rudiment. These cells were large, undifferentiated with basophilic cytoplasm and prominent nucleoli. They were found in the thymic primordium before its vascularisation and were thought to have migrated from the blood vessels into the surrounding mesenchyme, penetrating its basement membrane and entering the epithelium. 24-48 hours after injection, smaller labelled cells were found in the thymus that resembled large and medium lymphocytes; furthermore labelled dividing cells were also observed in the thymus (Moore and Owen, 1971).

It is not known for certain whether these stem cells are pre-committed to the T-lymphocyte lineage of differentiation before they reach the thymus or whether they are still multipotential. There is evidence that multipotential stem cells exist in the foetal mouse thymus at 12 days of gestation (Metcalf and Moore, 1971), while Barg et al (1978) report a peak of haemopoietic activity at

15 days, as indicated by the spleen colony assay (Till and McCullough, 1961). Once within the thymus, stem cells are not self-renewing as in their tissue of origin and their numbers are maintained by newly migrated stem cells. In the neonate and adult, stem cells continue to enter the thymus and are derived ultimately from bone marrow which becomes the primary haemopoietic organ. The marrow origin of thymic precursor cells in the adult has been shown in a number of studies. Brown et al (1953) and Kaplan et al (1954) noted that whole body irradiation of mice had led to the rapid involution of the thymus; rapid regeneration of the thymus could be achieved by shielding the thighs or by injection of syngeneic bone marrow cells, but not thymocytes. Harris et al (1964) using chromosome markers in parabiosed mice showed extensive traffic of cells between bone marrow and the thymus, and Gengozian et al (1957) were able to repopulate the irradiated mouse thymus with rat bone marrow. Cytological evidence of the relationship between marrow stem cells and lymphocytes was obtained by Wu et al (1968).

2. STRUCTURE OF THE THYMUS

Any study of lymphocyte differentiation in the thymus requires a consideration of its unique architecture that contributes to the microenvironment of the organ. The microscopic structure of the thymus has been well documented (Clark, 1973; Weiss, 1977; and Ham, 1977). Each of the two lobes is surrounded by a

The use of a species-specific chromosomal markers has enabled the evaluation of the contribution of epithelial, mesenchymal and blood-borne haemopoietic cells to thymic histogenesis in quail-chick chimaeras. The lymphoid population of the thymus was derived wholly from immigrant blood-borne stem cells which first seed the thymic rudiment at a precise stage of its development, characteristic for each of the two species. This initial influx being followed by defined, successive waves of cell inflow. The endoderm of the third and fourth pharyngeal pouches were determined to differentiate into the epithelial component of the organ (Le Douarin and Jotereau, 1975). Transfilter culture experiments have suggested that the onset of cell migration to the thymus takes place only when a chemotactic substance is produced by the thymus, its effect being exerted on blood-borne precursors. The elaboration of this attractant appears to be regulated by a feedback mechanism upon colonisation of the thymus by lymphoid precursors. The phases of thymic invasion last about 24 hours in the quail and occur every 5 days. Furthermore, it was found that cells that had already colonised the bursa of fabricius were not found to be refractory to the chemotactic influences of the thymus and were able to differentiate along the T-lineage (Le Douarin, 1980).

connective tissue capsule derived from mesenchyme which extends into each lobe as septae, dividing them into incomplete lobules. The epithelial cells are arranged in a sponge-like network, joined at their processes by desmosomes, thus being called reticular epithelial or reticulum cells. Lymphoid cells are found in much greater number in the outer part of each lobule, packed between the interstices of the epithelial cells, forming the cortex. The inner, medullary region has fewer lymphoid cells, thus the medullary epithelial cells are not widely separated as in the cortex and appear typically epithelial. In the outer cortex, the lymphoid cells are larger and appear as lymphoblasts; deeper in the cortex they are smaller and in the medulla they resemble peripheral small lymphocytes. Cell division of the large and medium cells to form small lymphocytes precedes their migration through the interstices of the epithelium into the medulla.

Branches of the subclavian artery enter the medulla through the septae, whereas only capillaries are found in the cortex. These are derived from arterioles located in the cortico-medullary junction, from where they ascend into the cortex forming anastamosing loops. They return to the cortico-medullary junction where they drain into the post-capillary venules of the medulla. By this vascular arrangement, the cortex lies "upstream" from the medulla, having first contact with blood-borne antigens whilst being isolated from any medullary humoral influences. The system also enables the possibility of a counter-current mechanism in the thymus. Clark (1963) found evidence of a continuous epithelium surrounding the capillaries of the cortex, the space

inbetween being filled with lymphocytes and macrophages. A basement membrane was seen to cover both the epithelial cells and the endothelium of capillaries. This arrangement is believed to constitute a blood-thymic barrier in the cortex which effectively precludes antigenic material in a capillary from reaching the developing lymphocytes. By injecting electron-opaque tracers, Raviola and Karnovsky (1972) have demonstrated that the blood-thymus barrier is impervious in the cortex but leaky in the cortico-medullary junction. The thymus lacks an afferent lymphatic drainage, but efferent lymphatics drain from the medullary region which also contains the medullary venules surrounded by a sheath of perivascular connective tissue. The small lymphocyte that enters the periphery is thought to leave the thymus in this region. Kotani et al (1966) have suggested some lymphocytes leave the organ via the perivascular lymphatic channels. However, Ernstrom et al (1965) cannulated thymic arteries and veins and observed a net gain of lymphocytes in the venous blood, suggesting a vascular route of exit.

3. DIFFERENTIATION IN THE THYMUS

The development of subpopulations of thymocytes has been extensively reviewed by Cantor and Weissman (1976) and Stutman (1978). The differentiation process from large stem cell to medullary small lymphocyte is accompanied by the acquisition of surface antigens, loss of sensitivity to glucocorticoids, an increase in responsiveness to the mitogen Phytohaemagglutinin (PHA),

and a decrease in thymus specific antigenicity. The above changes occur in concert with successive cell divisions. Administration of ^{3}H TdR parenterally or intrathymically shows the dividing cells are almost wholly large and medium lymphocytes in the cortex. If pulse-labelling techniques are used, the label is found to shift from large and medium cells to small lymphocytes; nearly 95% of the small lymphocytes being labelled by 2-4 days, therefore they are derived from labelled precurosrs in situ (Craddock et al, 1964; Metcalf and Wiadrowski, 1966; Fathman et al, 1975; and Weissman, 1973). However there is evidence that a population of self-generating cells exist in the medulla (Clark, 1968 and Shortman and Jackson, 1974).

The cytolytic effect of glucocorticoids on lymphoid tissue has been known for some time (Santisteban and Dougherty, 1954). Its effects are most apparent in the thymus where it causes lymphocytolysis within hours and leads to a dose dependent organ weight loss. There is a subpopulation in the medulla that appears to be cortisone resistant, and which lacks the surface antigenic determinants of more mature thymocytes (Schlesinger and Golokai, 1967). These cells are thought to be reactive in the graft versus host reaction (GVHR) (Cohen et al, 1970; and Blomgren and Andersson, 1969). Weissman (1973) demonstrated these cortisone resistant medullary cells to be derived from the cortisone sensitive precursors of the cortex. By locally applying ^{3}H TdR to the thymus capsule, he showed labelled cortical cells were sensitive to cortisone but their labelled medullary descendants were not.

During differentiation within the thymus, thymocytes express the following serologically defined surface antigens:- Ia, Thy 1, TL, GIX, MSLA, Th.B and the antigens of the Ly series (Reviewed by Raff, 1971 and Schlesinger, 1972). The Thy.1 antigen is found on thymocytes (Reif and Allen, 1964) and on thymus derived peripheral lymphocytes in either of two allelic forms, Thy.11and Thy 1.2 (Raff, 1969). The H.2 antigens coded by the major histocompatibility complex (MHC)are present on several tissues. The thymus leukaemia (TL) antigens are coded by a set of 4 linked genes either singly or in combinations, in certain strains of mice (Boyse and Old, 1969). Bone marrow stem cells possess H-2 antigens but lack Thy.1 (Ritter, 1978); the first cells to seed the thymus during ontogeny or which repopulate a thymus graft, lack thymus specific antigens (Owen and Raff, 1970). Later, smaller cells are seen which express high densities of Thy.1 and TL. Fractionation of thymocytes ondensity gradients of between 19-35% bovine serum albumin (BSA), separates cells into two broad bands. The majority of cells equilibrate between 25-29%; these denser cells have high Thy 1 and are TL positive, have low H.2 antigens and are cortisone sensitive. A more buoyant fraction (19-25%) were low Thy 1, TL negative, high H.2 and were cortisone resistant (Colley et al, 1970; Konda et al, 1973; and Levey and Burleson, 1972). These buoyant cells were effective in the GVHR, showed mitogen and mixed lymphocyte reactivity and migrated preferentially to lymph nodes, these properties being associated with peripheral T lymphocytes (Blomgren and Andersson, 1969 and 1971; Cohen et al, 1970; and Leckband and Boyse, 1971).

PHA responsive medullary thymocytes have been considered to be the immediate precursors of peripheral immunocompetent T lymphocytes. However, there is evidence that a population of cortisone resistant medullary thymocytes which are PHA responsive may never leave the thymus. They have an estimated life-span of at least 217 days (Elliott, 1973). Additional evidence for the medullary small lymphocyte not being the immediate precursor of immunocompetent peripheral T lymphocytes comes from passive transfer experiments. Spleen cells from lethally irradiated animals previously injected with syngeneic thymocytes or lymph node cells were tested for mixed lymphocyte reactivity against allogeneic cells. In animals reconstituted with thymocytes and cortisone resistant thymocytes, mixed lymphocyte reactivity was only seen after a period of 3-4 days, whereas lymph node cells were reactive within 24 hours of transfer. As cortisone resistant thymocytes are considered to be reactive in the mixed lymphocyte culture (MLC), these cells that migrate to the spleens of irradiated hosts must represent an immature subpopulation that requires further maturation in the spleen or periphery (Mosier and Cantor, 1971).

The differentiation from stem cell to T lymphocyte is wholly dependent on the thymus; this dependence is best illustrated in the congenitally athymic "nude" mouse that is homozygous for a recesssive, pleiotypic gene (nu/nu). These animals lack T lymphocytes and are unable to manifest a cell mediated immune response (Pantelouris 1968 and 1971; De Sousa et al, 1969; and Wortis, 1971a). It is thought that these mice have a defect in the

development of the epithelial component of the thymus (Wortis et al, 1971). In early embryonic life, a vestigial thymic rudiment appears but does not become lymphoid and remains epithelial (Cordier, 1974 and Owen et al, 1975). However, there have been claims that if the thymic rudiment is removed at 13 days of gestation and cultured, homozygous nu/nu thymuses become indistinguishable from heterozygous nu/+ thymuses in their capacity for lymphopoiesis and to generate GVH reactive cells (Chakravarty et al, 1975). The spleen and bone narrow of nu/nu mice possess pre-thymic cells which are capable of repopulating the thymus and ultimately the thymus-dependent areas of peripheral lymphoid organs, if thymuses from neonatal donors are grafted in these animals (Loor and Kindred, 1973; Pritchard and Micklem, 1973; Wortis et al 1971b and De Sousa and Pritchard, 1974). The immune reactivity of these thymus-derived host lymphocytes is markedly greater than that of untreated controls but lower than that of normal mice (Kindred and Loor, 1974 and Loor et al, 1976). The restorative effects of thymus grafting can only be achieved if a free thymus graft is used, and not if enclosed in a cell-impermeable diffusion chamber (Pierpaoli and Besedovsky, 1975). Thus traffic through the thymus is a pre-requisite to generate immunocompetent T cells. The age of the grafted donor thymus is significant, thymuses from adult animals did not restore immune reactivity in nu/nu mice (Radov et al, 1975). Adult and non-lymphoid, epithelial grafts were poorly repopulated by host cells (Loor and Hagg, 1977).

The Lyt markers which specify functional subclasses of peripheral T cells, are considered to be acquired in a stage-specific manner in distinct lineages of thymocytes. Specific antisera to these surface antigens therefore provide a probe for analysing T cell heterogeneity and intrathymic maturation.

It is thought that Lyt 1 cells comprise the helper and MLR reactive cells, and Lyt 2 the suppressor and cytotoxic populations. A population that expresses both antigens ie. Lyt 1,2 is considered to represent precursors of cytotoxic and suppressor cells. The minority of immunocompetent cells in the thymus are considered to have the distribution of markers characteristic of peripheral T cells. By direct injection of fluorescence intrathymically, the phenotypic distribution of labelled migrants in peripheral lymphoid organs were studied. About 75% of thymus migrants were Lyt 1 and 25% were Lyt 1,2. Thus, it would appear that the phenotypes of T cells for the Lyt antigens at least are determined within the thymus (Scollay et al, 1978). Furthermore, the same experimental technique has shown that the medulla is not the primary source of thymic migrants as was once thought. The emigrant cells themselves were mainly cortisone-sensitive cortical cells (Scollay et al, 1980).

With monolonal antisera raised to these antigens, it has been possible to detect both Lyt 1 and Lyt 1,2 cells early in all subpopulations of thymocytes, including the early subcapsular lymphoblasts. These findings suggest at least two separate lineages; the Lyt 1,2 class not being a precursor of the Lyt 1 subclass. Likewise, the ThB antigen was found to be on all subclasses of cells in the thymus. Therefore, it appears that the thymocyte population is heterogenous even within the earliest precursor population (Scollay and Weissman, 1980).

4. HUMORAL INFLUENCES OF THE THYMUS

The possibility that the thymus may be an endocrine gland has long been suspected by Maximow and by Gregoire (Reviewed by Metcalf and Moore, 1971). Maximow postulated that the epithelial cells of the thymic medulla somehow attracted wandering lymphoid cells (wanderzellen) and stimulated their proliferation within the thymus. Gregoire speculated that this "lymphoepithelial symbiosis" was mediated by a hormonal mechanism. More recent evidence to substantiate the endocrine hypothesis came from ablation-replacement experiments, where neonatally thymectomised (NTx) mice were immunologically restored when implanted with thymus grafts. The two alternative but mutually inexclusive interpretations were that host cells became immunocompetent by traffic through the grafted thymus, or the graft secreted a humoral factor whose target was the host cells (Dalmasso et al, 1963; Miller and Osoba, 1963 and Harris and Ford, 1963 and 1964). The humoral hypothesis gained further credance when the restorative effect was mediated even when the graft was enclosed in a cell impermeable diffusion chamber (Levey et al, 1963a and b; Law et al, 1964 and Osoba and Miller, 1963 and 1964). Indirect evidence from a physiologically normal situation was obtained when NTx CBA female mice were mated with normal males carrying the T6/T6 chromosome marker. After the birth of their litters, the immunological responses of the NTx females were similar to sham Tx and pseudopregnant controls, although lymphocyte levels were not significantly different before and after pregnancy. As T_6 marked cells of foetal origin were not found in the NTx females,

the remedial effect could be attributed to a humoral factor elaborated by the foetal thymus in utero, which crossed the placenta (Osoba, 1965 and 1973).

It was observed that thymus grafts in diffusion chambers were not able to restore the immunocompetence of NTx mice if treatment was delayed for 40 days after Tx. It was proposed that a peripheral population of post-thymic precursors of T cells were sensitive to the humoral influences of the thymus; furthermore, they were dependent on the intact thymus for their renewal and maintenance of numbers. Further evidence for this hypothesis was obtained when haemopoietic tissue from adult, newborn and early embryos was injected into NTx animals containing either free thymus grafts or ones in diffusion chambers. Embryonic haemopoietic cells required a free graft, whereas adult and newborn cells which presumably contained post-thymic cells did not require cellular contact with the grafted thymus to restore the NTx hosts (Reviewed by Stutman, 1975 and 1977).

Several laboratories have isolated and characterised a number of polypeptides from the thymus (Reviewed by Bach and Carnaud, 1976). The most notable of these are thymosin (A.L. Goldstein et al, 1972), thymopoietin (G. Goldstein, 1974), thymus humoral factor (THF, Small and Trainin, 1967) and serum thymic factor (TF, Bach et al, 1972). Thymosin has been purified from several mammalian sources, and its biological activity has been described by A.L. Goldstein (1975) and by Low and A.L. Goldstein (1979). It is a homogenous protein of approximate molecular weight (m.wt.) 1200 daltons (D). Earlier partly purified extracts were shown to

reduce incidence of wasting disease (Asanuma et al, 1970; A.L. Goldstein et al, 1972), stimulate lymphocytopoiesis (Klein et al, 1965 and A.L. Goldstein et al, 1966) and enhance the rejection of skin grafts and tumours in immunodeficient mice (A.L. Goldstein et al, 1970 and Zisblatt et al, 1970) and induce thymocyte specific antigens to be expressed on spleen and bone marrow cells (Scheid et al, 1973). After pre-incubation with thymosin, thymocytes from NTx mice showed a dose-related response in the MLC. Lymph node cells from these mice previously treated with thymosin in vivo exhibited a marked increase in ConA responsive ness (A.L. Goldstein, 1975). Some restoration of the immunodeficiencies of adult NZB mice was achieved by prolonged thymosin administration. They regained their ability to reject skin allografts, respond in the MLC and raise antibodies to SRBC (Gershwin et al, 1974). The number of E-rosette forming cells in the lymphocytes of patients with immunodeficiency diseases could be increased upon incubation with thymosin in vitro (Warra and Ammann, 1975).

Thymopoietin, a polypeptide of m.wt. approx. 5000 D has been purified from bovine thymus. It induces the expression of Thy. 1 and T L antigens and mitogen responsiveness in pre-T cells in the spleen and bone marrow (Basch and G. Goldstein, 1974 and 1975 and Scheid, 1975). When administered to animals with experimentally induced autoimmune thymitis, a condition associated with myasthenia gravis, it improves neuromuscular transmission (G. Goldstein, 1974). A portion of the molecule with biological activity has been synthesised chemically (Schlesinger et al, 1975).

Komuro et al (1975) demonstrated that bone marrow and spleen cells induced to express TL and Thy 1 antigens could still repopulate the thymus of irradiated animals. This finding suggests that the target cell for the hormone is the pro-thymocyte, rather than a post-thymic cell.

Thymic humoral factor (THF) is an acidic polypeptide, m.wt. approx. 3000 D, prepared from bovine thymus (Reviewed by Trainin et al, 1975). Trainin and co-workers earlier demonstrated that cell free extracts of thymus were capable of restoring the ability of NTx mice to reject skin allografts (Trainin and Linker-Israeli, 1967). THF increased the cytotoxic activity of lymphoid cells to syngeneic tumours in vivo and in vitro (Garnaud et al, 1973). There is evidence that THF restores the GVH activity of spleen cells and their primary antibody response to sheep erythrocytes (SRBC) in NTx mice (Trainin et al, 1969; Trainin and Small, 1970; Lonai et al, 1973 and Rotter et al, 1973).

Serum thymic factor (TF) has been detected in human and animal sera by a T cell-SRBC rosette assay (E-rosettes) and is the smallest of these factors, m.wt. approx. 1000 D (Reviewed by Bach, 1975). The sensitivity of rosette forming cells (RFC) to azathioprine (AZ) a purine analogue has been used as a measure of T cell maturity (Bach et al, 1969). Sensitivity to anti-Thy. 1 serum and AZ of spleen RFC was lost within one week of adult thymectomy (ATx). RFC from spleens of ATx mice were rendered sensitive to anti-Thy. 1 and AZ by in vivo or in vitro treatment with thymic extracts. This activity was also found in normal mouse serum but not in sera from Tx or nude mice (Bach et al, 1971, Bach and Dardenne, 1973 and Dardenne and Bach, 1973).

It is of course possible that these various peptides isolated at different laboratories may be subunits or fragments of a single protein, each showing some activity in the various assays used. Several pharmacological agents have been found to mimick the effects of thymic hormones. Of these, c.AMP, its derivative dibutyryl c.AMP and agents that elevate the levels of endogenous c.AMP such as theophylline, isoproterenol and β-andrenergic agonists such as adrenaline were effective (Scheid et al, 1973 and 1975; Singh and Owen, 1975 and 1976).

5. THE THYMUS IN VITRO

As described previously, some degree of differentiation in pre-T cells was possible by treatment with thymus derived factors. However, traffic through the thymus was essential for complete immunological restoration. There have been numerous investigations on the effect of co-culturing putative T cell precursors with monolayers of thymus in vitro. The rationale being, these conditions would approximate the in vivo thymic microenvironment by permitting cellular interactions between T cell precursors and the reticulo-epithelial cells of the thymus. If the hormonal influences of the organ were normally exerted locally, that criteria would be possible in this system. The epithelial origin of thymic hormones has been demonstrated morphologically by Clark (1973) and functionally by Dardenne et al (1974). Administration of a rabbit antiserum specific for mouse thymic epithelial cells resulted in specific

changes in the ultrastructure of these cells, and a decrease in serum thymic factors (Garaci et al, 1978). It has been generally assumed that the cells cultured as monolayers from thymic explants are epithelial in nature. They have been described as large polygonal cells with large nuclei containing dispersed chromatin and dense nucleoli. Their vacuolated cytoplasm is stained by the periodic acid-Schiff reaction (PAS) and contains refractile granules. A rough endoplasmic reticulum and well developed golgi apparatus are apparent in electron micrographs of cultured cells. There are some reports that these cells are joined at their processes by interconnecting demosomes (Wekerle et al, 1973; Waksal et al, 1975 and Papiernik et al, 1975).

Mosier and Pierce (1972) cultured mouse thymocytes on monolayers of supporting cells derived from thymus or from splenic adherent cells. Following co-culture with thymus epithelium, mitogen responsiveness, MLC reactivity and helper function increased markedly. Thymocytes incubated with splenic adherent cells were more proliferative in both stimulated and unstimulated cultures, but only expressed an intermediate level of helper activity. There does not appear to be a species barrier in the potential for thymic epithelium to induce differentiation in xenogeneic pre-T cells. Wekerle et al (1973) found that spleen cells from thymus-deprived mice had increased Con A and GVH reactivity after co-culture with rat thymus epithelium. Waksal et al (1975) reported similar observations, rat epithelium being as effective as that from mouse. Papiernik et al (1975) reported that on co-culture with human thymic epithelium, RFC from ATx

mice become sensitive to inhibition by anti-Thy. 1 serum. The effects could still be obtained when target cells were enclosed in millipore chambers. Willis-Carr et al (1978) reported an increase in the number of E-rosette forming cells among the bone marrow lymphoid cells of humans after exposure to monolayers or supernatants from human and monkey thymus. Longer exposures to washed monolayers were needed, suggesting that induction was caused by some factor released into the supernatant. Monolayers were more effective in inducing differentiation in lymphocytes from patients with immunodeficiency diseases than supernatants, suggesting the necessity for cellular contact as a primary differentiation event. Sato et al (1976) isolated a pre-T cell, bearing a cell surface brain associated T antigen (BAT). These BAT positive cells could be induced to both Concanavalin A (Con A) and MLC reactivity after culture on thymus epithelial cells.

Some investigators have demonstrated functional maturation of pre-T cells by treatment with supernatants from monolayers alone. Kruisbeek et al (1977 and 1978) showed that supernatants from rat thymic epithelial cultures (TES) increased mitogen induced proliferation of cortisone sensitive rat thymocytes. In the presence of TES, nude mouse spleen cells showed enhanced in vitro antibody production to SRBC. Oosterom et al (1979) demonstrated that conditioned media from human thymus epithelial cultures enhanced mitogen stimulation of thymocytes and spleen cells.

The possibility that traffic through the thymus is necessary to enable humoral factors elaborated by the thymus to act at short range is supported by the findings of Burleson and Levey (1971).

They excised the thymus and aortic arch of rats and mice and set up the whole organ in culture, with cannulation of the descending aorta. This system allowed perfusion of the thymus through its intact arterial supply. Spleen cells from NTx mice and bone marrow stem cells separated on gradients were perfused in vitro through the thymus. The recovered cells exhibited some GVH activity in the Simonsen assay.

Morphological characterisation of the thymic monolayers as epithelial in these studies were generally presumptive. Extended culture of thymus in vitro has promoted growth of mast cells and striated muscle (Csaba et al, 1960; and Wekerle et al, 1975). Macrophages are a distinct cell type of the normal thymus; however recent evidence indicates that the predominant cells in thymic monolayers are in fact macrophages. Their radiosensitivity characteristics are more typical of macrophages than epithelium. By histochemical staining, their cytoplasm has been shown to possess non-specific esterases and they are actively phagocytic (Watkins and Sharp, 1978; Jordan and Crouse, 1979a; and Jordan et al, 1979a). It is interesting in this light to consider the earlier findings of Van den Tweel and Walker (1977), these investigators reported increased mitogenic responses in thymic lymphocytes after co-culture with macrophages from guinea pig peritoneal exudates. Beller and Unanue (1978) co-cultured thymocytes with gradient purified thymic macrophages and detected increased expression of H.2 antigens, decrease in TL and acquired ability to respond in the MLC. Furthermore, a factor from macrophage culture media exhibited some differentiation inducing activity.

It might be contended that the thymus cultured as monolayers might not provide the necessary microenvironment for differentiation, as it lacks the architecture of the intact organ. A tenable means of overcoming these limitations is made possible by the culture of thymic explants in organ culture. The embryonic mouse thymus between 14 and 16 days of gestation provides a useful system to investigate thymic lymphopoiesis. The size of the organ makes it suitable for organ culture, and the system can be studied in isolation without the complication of cellular migration to and from the organ. It is preferable as a source of thymic epithelium as it contains fewer lymphoid cells which are yet undifferentiated; at 14 days, the foetal thymus contains large, basophilic cells.

The organ culture system has been utilised by several investigators. If the early embryonic organ is explanted and cultured, differentiation proceeds and lymphoid cells bearing the TL antigen (Mandel and Kennedy, 1978) and mitogen and MLC reactive cells are generated (Robinson and Owen, 1976 and 1977). If grafted back into a syngeneic host after a period of culture, the organs are still able to be repopulated by host blood-borne stem cells (Mandel and Russell, 1971). Using a similar system, responsiveness to Con A, leukoagglutinin (LA), MLC activity and cytotoxicity to target cells was acquired in vitro (Tufveson et al, 1976 and Juhlin et al, 1977).

Experimental transplantation of thyroid tissue across the histocompatability barrier without rejection appears to be possible, if the tissue is cultured in oxygen beforehand. The loss of antigenicity was thought to be due to selective loss of passenger

leucocytes, which were considered essential as an allogeneic stimulus for sensitisation of the recipient's lymphocytes. Furthermore, passenger leukocytes in these cultured allografts were found to be particularly susceptible to killing by hyperbaric oxygen (Lafferty et al, 1975; Talmage et al, 1976; and Talmage and Dart, 1978). The technique has been applied to thymic tissue before transplantation, to allogeneic and xenogeneic hosts as well as nude mice, after organ culture. The grafts were shown to be repopulated by host cells without rejection (Schulte-Wissermann et al, 1978 and Hong et al, 1979). There have been other reports in the literature of methods of depleting passenger leucocytes or inactivating their effect on host sensitisation in organ grafts, prior to transplantation. Opelz and Terasaki (1974) suppressed the stimulatory activity in the MLC of lymphocytes maintained at 22°C. Based on this finding, Lacy et al (1979) reported that survival of isolated rat pancreatic islets in an allogeneic recipient was prolonged after a period of culture at 24°C. Jordan and Crouse (1979b) found that 14 day mouse foetal thymus cultured at 24°C for 7 days lost its lymphoid cells, while growth of the epithelial component was maintained. The tissue has been shown to be functionally viable by subsequent transplantation under the renal capsules of syngeneic mice, where it is repopulated by lymphoid cells of host origin.

6. THE T-PRECURSOR CELLS

The immunocompetent, peripheral T cells are thought to be comprised of several subsets, each with one or more specific

immune function (Reviewed by Cantor and Boyse, 1977). Similarly the thymocytes themselves are considered to be a heterogenous population with regard to their physical properties, surface antigenic determinants and multiple intrathymic differentiative pathways (Huber et al, 1976) and reviewed by Droege and Zucker (1975) and Cantor and Weissman (1976). In the literature, the terms stem cells, pro-thymocytes, pre-T cells and T precursor cells are often interchangeably used to define a population of cells capable of further differentiation under thymic influence. There is evidence that this population itself may be as heterogenous as the products of its differentiation process. The subject is further complicated by the multiple tissue locations of these cells; the bone marrow and spleens often being used as a source of these cells for experimental studies. The bone marrow is very heterogenous in its composition, with functional compartmentation of its cell populations. It is a primary site of lymphopoiesis as well as a transient location of recirculating mature cells. The bone marrow lymphocytes and transitional cells have been reviewed by Rosse (1976). The small lymphocytes of the marrow are generated de novo from transitional cells by a comparatively short production pathway of 2-3 mitoses, whereas 8-9 mitoses are needed in the maturation of thymus cells (Yoffey et al, 1978). By the use of autoradiography and immunofluorescent staining of B and T cells, Rosse and Press (1978) demonstrated that the majority of marrow lymphocytes are rapidly renewed B cells and null cells in guinea pigs and mice. The rate of B cell turnover in marrow exceeds

that in spleen and lymph nodes, the B cells in these two secondary organs being replaced continuously by lymphyocytes derived from the marrow. Osmond and Nossall (1974a and b) reported that many of the small lymphocytes of the murine bone marrow have readily detectable surface immunoglobulin (Ig) molecules, while those without were neither Thy 1 positive, thymus dependent or recirculating cells. The Ig bearing cells were predominantly small, non-dividing lymphocytes. In addition to surface Ig, marrow lymphocytes have been shown to have Fc and complement receptors (Yang and Osmond, 1979).

The presence of immunocompetent thymus derived cells in the marrow, capable of causing a GVHR has been demonstrated. These cells could be isolated by centrifugation on discontinuous BSA gradients from the spleen colony forming unit (CFU-S) stem cells (Burleson and Levey, 1972 and Dicke et al, 1968). Velocity sedimentation of bone marrow showed that the GVH reactive cells had a sedimentation velocity of 3 mm/hr. at 4°C and unit gravity; whereas the CFU-S sedimented at 4 mm/hr. (Phillips and Miller, 1970). Osmond and Yoshida (1970) incubated enriched bone marrow lymphoid cells with PHA, pokeweed mitogen (PWM), keyhole limpet haemocyanin (KLH), SRBC and allogeneic lymphoid cells in vitro or within chambers intraperitoneally. They observed blastogenic transformation and proliferation of these cells by 3-4 days.

While the above T-dependent functions can be attributed to recirculating thymus derived cells in the bone marrow, there is evidence that marrow lymphocytes behave atypically in this regard.

Blomgren and Svedmyr (1971) reported the thymic independence of PHA reactive cells in the regenerating marrow of lethally irradiated, bone marrow protected mice. PHA response first appeared in the cells of the bone marrow and later in the other lymphoid organs. The reactivity of these marrow cells was not sensitive to treatment with anti-Thy 1 + C^1. Ropke (1977a) reported increased numbers of Thy 1 bearing cells in the marrow of NTx mice or nude mice in contrast with normal animals. Cohen and Patterson (1975) found that Con A induced small lymphocytes in the marrow into lymphoblasts expressing Thy 1 and TL antigens, whereas spleen cells only expressed Thy 1 and not TL. Thymosin, c.AMP and dibutyryl c.AMP were also effective in this induction, the target cell was thought to be a pre-thymic cell. Cohen and Fairchild (1976) demonstrated these pre-T cells of the marrow were synergistic with mature cells and cortisone resistant thymocytes to enhance the latter's response to Con A. Nude spleen cells were also shown to be effective in this synergy. Basch et al (1978) have characterised the cells committed to thymocyte differentiation as a population bearing surface antigens common with mouse brain tissue. They are found among the null cell population of spleen and bone marrow and contain an unusual enzyme, terminal deoxyribonucleotidyl transferase (Tdt) in the marrow but not spleen.

El-Arini and Osoba (1973) isolated the pre-T cells in the marrow from mature T cells and CFU-S by buoyant density centrifugation. The mature, thymus derived, Thy 1 positive lymphocytes which were reactive in the GVHR and MLC banded at a density of 1.069 g/cm^3. Cells from the lighter density

region 1.050-1.059 g/cm^3 generated immunocompetent cells on repopulation of the spleens of irradiated isogeneic recipients, this fraction was thought to contain the CFU-S stem cells. The pre-T cells have an intermediate modal density of 1.064 g/cm^3. In a series of experiments on the kinetics and characterisation of PHA reactive cells of the bone marrow, Press and Rosse (1977a, b and 1978) and Press et al (1977) demonstrated that marrow lymphocytes purified on sucrose density gradients could be induced to respond to PHA after prolonged incubation with high doses of the mitogen. Lymphocytes from nude mouse marrow were identical in their response to those from normal mice. The responding cells were characterised as precursors of T cells, indirectly by use of specific antisera; their reactivity was not affected by pre-treatment with anti-Thy 1, anti-thymocyte, anti-brain, anti-IgM or anti-mouse gamma globulin sera. Furthermore, during the blastogenic response, the "null" cells acquired T cell surface antigens, demonstrated by fluorescent anti-brain sera, the effect was reproducible even after pre-treatment with anti-brain + C'. By previously injecting the animals with ^{3}H TdR, it was possible to selectively label the cells with slow and rapid turnover rates. Autoradiographic analysis of PHA stimulated cells identified the blastogenic cells of the marrow as derived from the rapidly renewed population.

Gorcynski and MacRae (1977a) used Thy 1 depleted marrow and spleen cells to reconstitute the immune functions in Tx and intact lethally irradiated syngeneic recipients. They obtained evidence for both a pre-thymic and post-thymic precursor population in both organs. The post-thymic cells reconstituted

limited T cell functions early in Tx animals; the pre-thymic precursors generated first a T suppressor (T^S) and later a helper (T^H) population for cell mediated lympholysis (CML) in the intact animal. All these functions were first seen in large cells which reverted to small cells. In a separate study, these authors demonstrated that post-thymic precursors were able to develop into mitogen reactive cells in culture, a proportion of these gave rise to MLC and CML reactive cells. A similar pool of precursors, but which were Thy 1 negative generated cells with helper function for antibody responses. T^S cells were also generated after 4 days in culture from larger cells which were largely Thy 1 negative. By velocity sedimentation of T-depleted marrow and spleen cells, fractions which could generate precursors of cytotoxic lymphocytes (CTL), of Con A responding cells and those with helper function for CML could be isolated. All these subpopulations were thought to be derived from a post-thymic precursor pool, whereas T^S cells were from pre-thymic precursors (Gorczynski and MacRae, 1977b). In a further study, these authors investigated the ability of pre and post-thymic precursors to differentiate on feeder layers derived from thymus and with supernatants of these. The slow sedimenting, small post-thymic precursors differentiated as effectively under both stimuli. The rapidly sedimenting, large pre-thymic precursors needed prolonged co-culture with feeder cells to induce precursors of CTL, helper cells for CML and mitogen responsive cells. However, after 5 days in culture, further differentiation was possible with supernatants alone, this latter step being mediated by a second "soluble signal" (Gorczynski and MacRae, 1979).

Although there is much evidence for the thymus repopulating capacity of bone marrow haemopoietic cells, there is some doubt whether they serve as stem cells for all the differentiated blood cells ie: whether stem cells with restricted differentiative capacity exist. PHA transformed blasts of T cells in patients with chronic myeloid leukaemia (CML) do not possess the Philadelphia chromosome marker found in the granulocytic, megakaryocytic and erythroid lineages, thus indicating the possibility of a separate lymphocytic lineage (Whang et al, 1963).

Two phenotypic variants with different electrophoretic mobilities of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) can be detected and are coded by the X-chromosome (Fialkow, 1977). Using this marker, it has been possible to trace a clonal origin for all blood cell lineages except for T cells, during the remission phase of CML (Fialkow et al, 1978). However, Prchal et al (1978) using the same marker have found a common progenitor for lymphoid and myeloid cells in a patient with acquired idiopathic sideroblastic anaemia.

When a bone marrow inoculum containing radiation-induced chromosome aberrations was given to haemopoietic cell deficient W/W^{v} mice, the repopulation and generation of functional cell lineages could be determined. The presence of these unique chromosome markers were looked for in bone marrow, spleen and thymuses of such repopulated mice. A pluripotent stem cell was detected which shared the chromosome marker with myeloid, B and T cells. In addition, there was also evidence for two other restricted stem cells with distinct clones generating only myeloid and T cell lineages. Furthermore,

in a subsequent transfer of cells to an irradiated secondary recipient, the same tissue distribution of marked cells was obtained (Abramson et al, 1977). These data taken together, indicate a restricted haemopoietic stem cell, at least for the myeloid and T cell lineages.

7. KINETICS OF THYMUS CELLS

The earliest quantitative studies on the thymus indicated that the organ was a site of intense mitotic activity; Kindred (1940) observed that the cortex had a higher mitotic index than the medulla. Andreasen and Christensen (1949) compared the mitotic activity in the lymphoid organs of the rat by counting the mitotic figures in suspensions of isolated cell nuclei. The number of mitotic figures in the thymus being 4-6 times greater than that of lymph nodes and spleen, the mitotic activity was found to decrease with increasing age and following starvation. Saint-Marie and Leblond (1958) estimated that each small thymus lymphocyte was the product of a series of 8 consecutive reductive mitoses of large lymphocytes, their progeny continuing to divide into progressively smaller lymphocytes. They based their stem cell renewal theory on the erroneous assumption that each reticular cell in the cortex gave rise to large lymphocytes as well as maintaining the reticular cell population. Nakamura and Metcalf (1961) quantitated the mitoses in lymphoid cells of the thymus of C_3H mice from birth to the age of 7 months. The mitotic index was very high in the newborn, and after the first few weeks of life, decreased progressively over the following 6 weeks. Thus, exposure to foreign antigens did not seem to stimulate mitotic activity. The decrease in mitoses paralleled the age-involution weight loss of the organ, and continued to fall throughout the life of the animal. Pyknotic cells were seen in the thymus at all ages; the number of mitoses only exceeded the number of dying cells

during the period of thymus growth. This finding raised the possibility that most cells produced in the thymus never left the organ, but died in situ. Most mitotic cells were found in the cortex, the mean cell cycle times for large and medium lymphocytes being 6.8 and 8.2 hours respectively. Most small lymphocytes were non-dividing, but their population was replaced by the progeny of larger proliferating cells every 3.2 days (Metcalf and Wiadrowski, 1966). It is estimated that as many as 99% of the small lymphocytes produced in the thymus die locally, the surviving cells were thought to migrate to the periphery. However, Michalke et al (1969) reported an extensive migration of lymphocytes from the thymus of newborn mice, a fraction corresponding to a third of all lymphoid cells present being lost by the third day after birth. Because of the low pyknotic index at this age, the data indicated emigration of cells being the major cause of cell loss. In a later study, the thymic cortex was locally labelled with radioactive Iodo-deoxyuridine (^{125}I-UdR) in young adult mice, to quantitatively assess the fate of cortical thymocytes. More than 90% of the initial thymic radioactivity was lost after 8 days, only a few % of the total activity was detected in the spleen, mesenteric lymph nodes and femurs. This discrepancy could be explained by the death of the vast majority of newly formed thymocytes after a short life-span, only a small fraction of thymic migrants being longer lived (Laissue et al, 1976). Similar findings were reported by Joel et al (1977).

As proliferating thymocytes are not completely self-sustaining, they are dependent on the arrival of new blood-borne precursor

cells. The atrophy of the thymus with age could be accounted for by a reduced supply of stem cells and a change in the general hormonal balance of the animal after puberty. However, in the experiments of Metcalf (1961), old mice were grafted with neonatal thymuses; the grafts' mitotic indices were higher than that of the host's. Thymus grafts grew equally well in Tx and sham Tx hosts. When grafted with multiple grafts, each graft grew to the size typical of the strain and age of the grafted thymus, regardless of the age of the host. As both grafts and host thymuses were repopulated by host cells and shared the same hormonal and nutritive environment, the differences could only be attributed to the functional activity of the reticulum and some other non-lymphoid cells. The possible role of reticulum cells in the control of thymic lymphopoiesis was indicated by the observations of Metcalf and Ishidate (1961). They found a higher number of mitotic lymphocytes in contact with the reticulum cells than in other areas of the cortex. The reticulum cells were visually differentiated by their reaction with the PAS stain. Furthermore, the mitotic cells in contact with the PAS positive cells were mostly in the early stages of mitoses; it was speculated that the reticulum cells somehow initiated cell division. Metcalf (1964) postulated that the medullary epithelial cells could have a regulatory function on thymic lymphopoiesis, as they were unique to the thymus, their absence in lymph nodes accounting for the differences in mitotic activity in the two tissues. It was suggested that a secretory product of medullary epithelial origin somehow initiated mitosis in the cortex. The arrangement of arterioles in the thymus suggest a possible counter-current mechanism, whereby humoral secretions of the medulla could be

concentrated and made available in the cortex (Clark, 1973).

The findings of Bullough and Laurence (1960) of an epidermal mitotic inhibitor has prompted much investigation into other possible endogenous tissue-specific mitotic inhibitors or "chalones". Olsson and Claesson (1975) obtained a transient 40% reduction in the proliferation of thymus cells in vivo after a single injection of a water-soluble extract from mouse thymus. The thymus has been implicated in certain lymphoproliferative diseases; a lymphocytosis stimulating factor has been found in the plasma during such conditions, and is thought to be derived from thymus epithelium. It is found in higher concentrations in thymus of humans and mice with chronic lymphocytic leukaemia (CLL) and can be obtained from mouse thymus in tissue culture. When injected into neonatal mice, it cause a marked lymphocytosis (Metcalf, 1956).

Apart from the involution that accompanies senesence, a naturally occurring transient atrophy of the thymus occurs during pregnancy. This effect was first observed by Jolly and Lieure (1930) in guinea pigs and by Persike (1940) in mice, they noted that the thymus decreased in weight and at parturition could weigh as little as a fifth of normal controls. Pepper (1961) observed that the maternal thymus lost weight during pregnancy, continued to do so throughout lactation and hypertrophied after. These gross variations are accompanied by histological changes. Maroni and De Sousa (1972) observed that at 10 days of gestation, the murine thymus had a decreased number of lymphocytes in the cortex. Later in pregnancy, the cortex narrows and can become indistinct from the medulla. It is possible to attribute these changes to increases in corticosteroid levels during pregnancy and lactation.

However, the thymus was shown to lose about 70% of its weight while the lymph nodes weighed about 30% less. An earlier study by Santisteban and Dougherty (1954) demonstrated that the thymus was more sensitive than the lymph nodes to the effects of adrenal corticosteroids.

An immunological enigma that has proved largely inexplicable is why the mother does not mount an immunological reaction against the foetus and reject it as it would a homograft. Several hypotheses have been offered to explain this phenomenon, one of them being that the mother is immunologically inert. This state of unresponsiveness could possibly be mediated by the immunosuppressive action of increased adrenocortical hormones during pregnancy. Heslop et al (1954) found that skin homografts transplanted to rabbits late in pregnancy survived twice as long as in non-pregnant controls. Billingham (1951) showed that cortisone could be immunosuppressive when applied locally on a skin graft at dosages which were not effective when administered systemically. Thus the high levels of hormones secreted by the placenta could be sufficient to act locally in the in vivo situation. Similarly, human chorionic gonadotrophin (HCG) has been suggested as an immunosuppressant, as it is known to reduce the response of human lymphocytes to PHA in vitro (Hau, 1975).

The causes for these changes in the immunological potential of the mother may ultimately be traced back to the thymus, which itself undergoes marked changes in its cellular profile during pregnancy. These changes might explain the observations of Strelkauskas et al (1975) who found that during the first trimester

of pregnancy in humans, peripheral T and B cell levels were inverted and resumed their normal levels during the third trimester. The reduced T cell numbers early on in pregnancy could result in impaired cell mediated immunocompetence, with the establishment of tolerance. The increased numbers of B cells could facilitate the production of blocking antibodies, sensitised maternal lymphocytes and blocking antibodies were demonstrated by Hellstrom et al (1969) in mice.

The changes in the cellularity of the thymus during pregnancy could, in theory be caused by either a reduced proliferative activity of its cells or by a decrease in the entry of stem cells into the organ, or a combination of both. These alternatives could be at least partly resolved by a study of the cell kinetics of the thymus during pregnancy or some other involutional state. Fabrikant and Foster (1971) utilised ^{3}H TdR labelling indices in an investigation of the cell kinetics in mouse and rat thymuses. They conclude that the predominant mechanism which altered the cell population structure in the thymus appeared to be an increase in the size of the proliferating population during active thymus growth ie. through the addition of new stem cells and their progeny. Thymus involution occurred primarily through a decrease in the size of the proliferating population, the cellularity was not altered by significant differences in the cell cycle characteristics. Zaitoun et al (1979) used ^{3}H TdR labelling and stathmokinetic techniques to investigate the cell population kinetic profile of the normal mouse thymus as well as in animals treated with prednisolone, which causes marked thymic atrophy. Prednisolone had an inhibitory effect mainly in the S-phase and in G.1 . There

was a decrease in the cell birth rate and an increase in the apparent cell cycle time after treatment; the labelling index, mitotic index and the growth fractions were also decreased. They estimate that prednisolone treatment reduces the cell production rate by approx. 60% and the size of the proliferating pool by 40%. This would effectively cause a reduction in the output of lymphocytes from the thymus to nearly a quarter of normal amounts. Such a marked decrease in the generation of T lymphocytes caused by similar hormonal effects in vivo may well have significant consequences on the immunological reactivity of the pregnant animal.

8. The scope of this study

It is evident that the differentiation of multipotential stem cells to thymus-derived T lymphocytes occurs in more than one haemopoietic environment. Both hormonal influences and cell-to-cell interactions exerted by the non-lymphoid cells of the thymus are believed to be involved in the processing of T-precursor cells, prior to further maturation in the periphery.

It was intended to dissect and study in isolation the microenvironmental influences of the thymic non-lymphoid cells on the maturation of T precursor cells in vitro. Thymic lymphopoiesis is influenced by a number of factors including the availability of stem cells, age and systemic hormonal influences. As the cellular profile of both the thymus and peripheral T cells

alters during pregnancy, it was intended to investigate whether these changes could be attributed to the proliferative capacity of thymic lymphocytes during this transient and physiologically normal condition.

MATERIALS AND METHODS

A. Thymus monolayer cultures

1. Primary cultures

Young adult male mice of the CBA strain (obtained from the Bute animal house breeding stock) were killed by ether asphyxia and their thymuses removed under asceptic conditions. The organs were immediately placed in cold RPMI 1640 medium (Flow) buffered to pH 7.4 with 10^{-2} M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) and minced with sterile instruments to fragments of 1-2 mm size. The tissue was then washed in medium and transferred to pre-wetted 25 sq cm culture flasks (Corning), each flask contained one thymus. The thymic fragments were left undisturbed for approximately 15 minutes to allow them to adhere to the plastic surface. 2 ml of RPMI 1640 or Eagles minimal essential medium (MEM, (Flow)) buffered to pH 7.4 with 0.04 % (w/v) sodium bicarbonate ($NaHCO_3$) containing 2 mmoles of glutamine and 10% heat inactivated foetal calf serum (HIFCS, both from Flow Laboratories) and 50 units each of penicillin and streptomycin (Difco) was added per flask. The flasks were gassed with a mixture of 95% air / 5% CO_2, stoppered and incubated at 37°C. After 24 hours, the medium was changed, the tissue fragments having adhered firmly to the flasks. 5 ml of fresh medium was added per flask, regassed and incubated at 37°C. The medium was changed every 3 days and the cultures examined under an Olympus inverted microscope with phase contrast.

2. Harvesting cells from monolayers

Calcium and magnesium free phosphate buffered saline (CMF-PBS) was previously warmed to 37°C. 0.25% trypsin was

made up in CMF-PBS contai ning 10^{-3} M EDTA (ethylenediamine-tetraacetic acid, BDH). Thymus monolayers from primary cultures approx. 1 month old were washed thoroughly with CMF-PBS; 2-3 ml of the trypsin solution was added per flask and incubated for approx 2 mins at 37°C. The enzyme solution was discarded and approx the same amount of fresh trypsin was added to the flasks and incubated further at 37°C with gentle agitation on a roller (Denley Instruments). The monolayers were periodically examined under the inverted microscope and when most of the cells had detached, the enzymic process was inhibited by adding approx 5 ml of RPMI-Hepes containing 20% HIFCS. Cells were resuspended by gentle aspiration through a pasteur pipette and transferred to 10 ml plastic tubes (Sterilin) and centrifuged in an MSE Chilspin centrifuge (Fisons) for not more than 10 mins at 200 G. The supernatant was discarded and the cell pellet gently resuspended and washed in medium containing serum X2 more. The cells were then resuspended in 5ml RPMI-Hepes containing 20% HIFCS and the viability determined by a trypan blue dye exclusion test. The cells were either used for making cytospin preparations for morphological examination or for further culture in vitro.

3. Subculture of monolayer cells

The resuspended trypsinised cells were adjusted to a concentration of approx 10^5 cells/ml and plated out in 25 sq cm tissue culture flasks as described above. The cells were allowed to adhere to the substrate by incubating at 37°C overnight. The supernatant containing the non-adherent cells was removed

and 5 ml of fresh medium containing serum was added. The cells were cultured as described previously.

4. Inducitve properties of monolayer cells

The culture medium (5 ml) from the flasks were changed every 3 days. This conditioned medium (CM) was collected at at these times and centrifuged at 200 G for 10 min and the cell-free supernatant retained. 10^7 Syngeneic thymocytes were incubated in the CM for 24 h at a conc. of 2×10^6/ml at 37°C. The cells were recovered, washed and set up in the microculture system with PHA, to be described later. Alternatively, 10^7 thymocytes were incubated in the culture flasks on the third day after medium change. They were recovered 24 h after incubation at 37°C and the monolayers rinsed with fresh medium. The cells were then similarly cultured with PHA.

5. Processing of monolayers for light microscopy

(a) Jenner-Giemsa stain

The culture medium from the flasks was discarded and the monolayers rinsed with PBS. Approx. 5 ml of absolute methanol (BDH) was added per flask and fixed for 10 mins. 5 ml of Jenner's stain (previously diluted, 45 ml Jenner's:113 ml buffered water pH 6.4) was added to the flasks and left for 5 min, then discarded and rinsed with buffered water. The monolayers were then stained with Giemsa (also diluted, 25 ml Giemsa:150 ml buffered water) for 10 min. The stain was discarded, rinsed with buffered water and dried in air.

(b) Periodic acid Schiff's stain

Culture flasks were washed with PBS for 15 min at 37°C and

fixed as before. A 0.5 % solution of periodic acid (HIO_4) was added for 5 min, then rinsed in several changes of distilled water. Schiff's reagent was added for 10-15 min and then rinsed with 2 x 3 min changes of distilled water. The flasks were then rinsed under running water for 5 min and counterstained with Harris' haematoxylin for 20-30 sec and further rinsed in running water for 5 min.

(c) Histochemical detection of non-specific esterase

The simultaneous capture method was used, the substrate α-naphthyl acetate (Sigma).

(1) Pararosaniline was made by dissolving 2 g of pararosaniline hydrochloride in 50 ml of 2M hydrochloric acid by heating gently, cooling to room temperature and filtering.

(2) Hexazotised pararosaniline was made by mixing 0.3 ml of (1) with freshly made 40% sodium nitrite ($NaNO_2$, BDH) and allowing to stand for 1 min. As the diazotate is unstable, this was made up fresh just before staining. The reaction mixture was made fresh and composed of:-

4 mg 2-naphthylacetate dissolved in 0.5 ml of methylene glycol monoethyl ether (Cellosolve, BDH) and mixed with 0.5 ml of (2) and 8.6 ml of (3) 0.15 M Sorensen's buffer at pH 7.6

Potassium dihydrogen phosphate (KH_2PO_4) - 9.07 g/l

Disodium hydrogen orthophosphate ($Na_2HPO_4 2H_2O$)- 11.9 g/l

The medium from the flasks was discarded and rinsed in PBS and monolayers fixed in situ with (4) 10% neutral formol calcium for 15 min. The fixative was made as:-

10 ml of a 40% solution of formalin

1 g of calcium chloride ($CaCl_2$): distilled water to 100 ml

The monolayers were dried in air for 1 h and then incubated with the esterase reaction mixture for 40 min, washed in buffer and counterstained with Mayer's haemalum for 5 min. They were then dried and viewed under an inverted microscope.

6. Cytocentrifuge preparations

Single cells prepared from trypsinised monolayers or from intact lymphoid tissue were examined morphologically on cytocentrifuge preparations. The cells were resuspended to a concentration of 10^5/ml in medium containing 20% serum, placed in chambers of a 'Shandon' cytocentrifuge and spun at approx. 100 G for 10 min. The smears were air-dried, fixed and stained as in 5(a).

7. Measurement of growth rate of monolayers

Monolayers of thymus cells were cultured as described previously and their rate of growth was estimated by counting the numbers of cells in the same randomly chosen locations at regular intervals over the culture period. Some attempt was made to classify the cell types on the basis of their size and shape. The cultures were observed from 2-4 days after start of culture till the individual areas observed became confluent.

8. Characterisation of monolayers

(a) Phagocytosis of yeast cells

A 1% suspension of baker's yeast (Fisher and Donaldson) was prepared in PBS and heat-killed in a pressure-cooker at 15 psi for 1 h. The medium from the culture flasks was discarded and 5 ml of the yeast suspension, pre-warmed to 37°C was added to

each flask. The flasks were incubated at 37°C for 1 h, and then the yeast suspension was discarded and the monolayers rinsed in PBS to remove any remaining extracellular yeast. The monolayer cells were then fixed in methanol and stained with Jenner-Giemsa. Alternatively, they were fixed with neutral formol calcium and used for histochemistry as described previously.

(b) Detection of Fc receptors on thymic monolayer cells

Rabbit anti-ox red cell serum (Rab α-ox) a kind gift from Dr. A.A. Nash (University of Cambridge) was heat inactivated at 56°C for 30 min, aliquoted and stored at -20°C. Ox red cells were obtained from the St. Andrews slaughterhouse, centrifuged at a 1000 G for 10 min, the plasma removed, resuspended and washed x 3 in PBS. It was stored at 4°C for a maximum of 2 weeks in Alsever's solution.

Ox erythrocytes were washed x 3 in PBS and made up to a 5% suspension. To 0.5 ml of this was added 20μl of the Rab α-ox sera and incubated for 40 min at room temperature. The antibody coated red cells were then washed x 3 in PBS to remove unbound antibody and resuspended in approx. 2.5 ml of RPMI-Hepes containing 2% HIFCS. The monolayers were washed thoroughly with PBS and a small volume of the antibody coated red cell suspension was added so as to just cover the monolayer. They were left for 1 h at room temperature and gently rinsed with PBS to remove the excess red cells. The monolayers were then fixed in methanol and stained with Jenner-Giemsa. For controls, peritoneal exudate cells (PEC) similarly cultured as monolayers were also treated as above to detect Fc receptors.

9. Electron microscopy techniques for monolayer cells

(1) Monolayers were fixed in glutaraldehyde/paraformaldehyde (BDH) for 1 h *in situ*.

(2) Washed in 0.08 M sodium cacodylate buffer (BDH) x 3 for 5 min each.

(3) Fixed with osmium tetroxide (OsO_4, BDH) in buffer for 1 h at room temperature.

(4) Washed in buffer.

(5) Dehydrated sequentially in 75%, 96% and x 3 changes of absolute alcohol for 15 min each. In the penultimate alcohol rinse, they were stained with methylene blue to detect the cells.

(6) Monolayers were treated with equal parts of absolute alcohol/epoxy propane (BDH), then treated with x 3 changes of epoxy propane for 15 min each, which dissolved the top of the plastic substrate thus dissociating the cells.

(7) The cells were collected in a glass vial and treated with equal parts of epoxy propane/araldite and left overnight with the lid slightly open to allow evaporation of the epoxy propane.

(8) The epoxy propane/araldite mixture was discarded and replaced with fresh araldite and then left for 6 h as above.

(9) The cells were then embedded by placing the vials in a 60°C incubator for 36-48 h to allow the resin to polymerise.

(10) Silver sections of thickness 0.05 microns were cut on a Reichert-Jung OMU3 ultramicrotome and electron micrographs taken on a Zeiss EM9S2 microscope.

Alternatively, cells were trypsinised from monolayers and pelletted by centrifugation. The cell pellet was then fixed and processed as before, except it was not stained with methylene blue.

B. Organ culture of foetal thymus

1. Source of tissue

Foetal thymuses from embryos of CBA mice at 14, 15 and 16 days of gestation were used in this study. Three young adult female mice were housed per cage with one male and checked daily for the appearance of vaginal plugs, which was considered as day 0 of gestation. The pregnant mice were killed by ether asphyxia, their uteri removed and the foetuses dissected under a Kyowa binocular microscope. The thymuses were removed under asceptic conditions and transferred to plastic petridishes (Nunc) containing cold RPMI-Hepes medium. The thymuses were dissociated from contaminating connective tissue and blood vessels and separated into their individual lobes.

2. The organ culture system

This was a modification of the method described by Trowell (1954). Intact foetal thymic lobes were placed on small pieces of 'millipore' filters approx. 4 sq mm in size, which were themselves supported on stainless steel grids of approx 1 sq cm. These were placed in small plastic petridishes (Sterilin), one grid per dish and 3 ml of culture medium was added so as to form a meniscus at the grid. The medium used was either RPMI 1640 or Waymouth's (Flow), bicarbonate buffered and containing HIFCS, glutamine and penicillin/streptomycin (as before). In some

protocols, serum-free medium was used. The petridishes were placed in larger petridishes (three per large dish) and these were stacked in an organ culture jar (Gallenkamp) which was gassed, sealed and incubated at 37°C in a moist atmosphere. The jars were gassed with either a mixture of 95% air/5% CO_2 or 95% O_2/5% CO_2 and the media was changed every 2-3 days. The thymuses were cultured for varying times but for not more than 14 days; the explants were then either transplanted under the renal capsules of mice or prepared for morphological examination.

3. Transplantation under renal capsules

The thymic explants after organ culture were transplanted under the renal capsules of syngeneic or allogeneic hosts. The recipients were anaesthetised by administering the drug Sagital (BDH) intraperitoneally (I/P) at a dose of 1.5 mg/mouse. The surgical procedure was kindly performed by Dr. A.C. Riches. An incision was made in the skins and a further incision through the body wall on the left of the abdomen. The left kidney was located and a longitudinal incision was made in the renal capsule with a sterile scalpel blade. One or two thymus lobes were then inserted under the capsular layer. The edges of the capsular incision were then reapposed. The kidney was repositioned on the posterior abdominal wall and the body wall incision was sutured with surgical silk (Ethicon). The skin incision was then closed with Michell clips. The animals were kept warm under table lamps until they revived. The clips were removed two weeks later and the animals sacrificed one month after the transplant. The kidneys were removed and the grafts examined and treated for

histology.

4. Injection of cells into thymic explants

Thymic explants after organ culture in 95% O_2/5% CO_2 were injected with concentrated suspensions of haemopoietic cells. Cells were obtained from the bone marrow of normal animals and those treated with the cytotoxic drug mustine hydrochloride (nitrogen mustard HN_2, Boots) at a dose of 4 mg/kg body wt. In addition, thymocytes from 15 day embryos were also used. The cells were loaded into injection needles manufactured from glass capillary tubing on a pipette apparatus with a platine spiral. This was prepared in two stages; at first a pipette with a diameter of 60-80 microns was obtained, followed by a second step to obtain a diameter of 10-20 microns. The injection needles were connected by a means of thin 'Portex' tubing to an 'Agla' micrometer syringe (Wellcome). No more than 1-2 μl of the cell suspension containing approx. 10^5 cells were injected into the explants.

5. Morphological examination

(a) Light microscopy: Paraffin sections

Standard histological procedures had to be modified because of the diminutive size of these explants. Foetal thymic explants were fixed in Bouin's solution for 3-4 h, then transferred to 70% ethanol. They were treated sequentially to 96% ethanol, 2 x changes of absolute ethanol and chloroform, each of 15 min duration. A further incubation in fresh chloroform for 30 min was followed by transfer to paraffin wax for 45 min under vacuum. The explants were then embedded in fresh paraffin wax and mounted in blocks. Sections of 5 microns thickness were cut on a Leitz

microtome, mounted on microscopical slides and stained with haematoxylin and eosin (H and E).

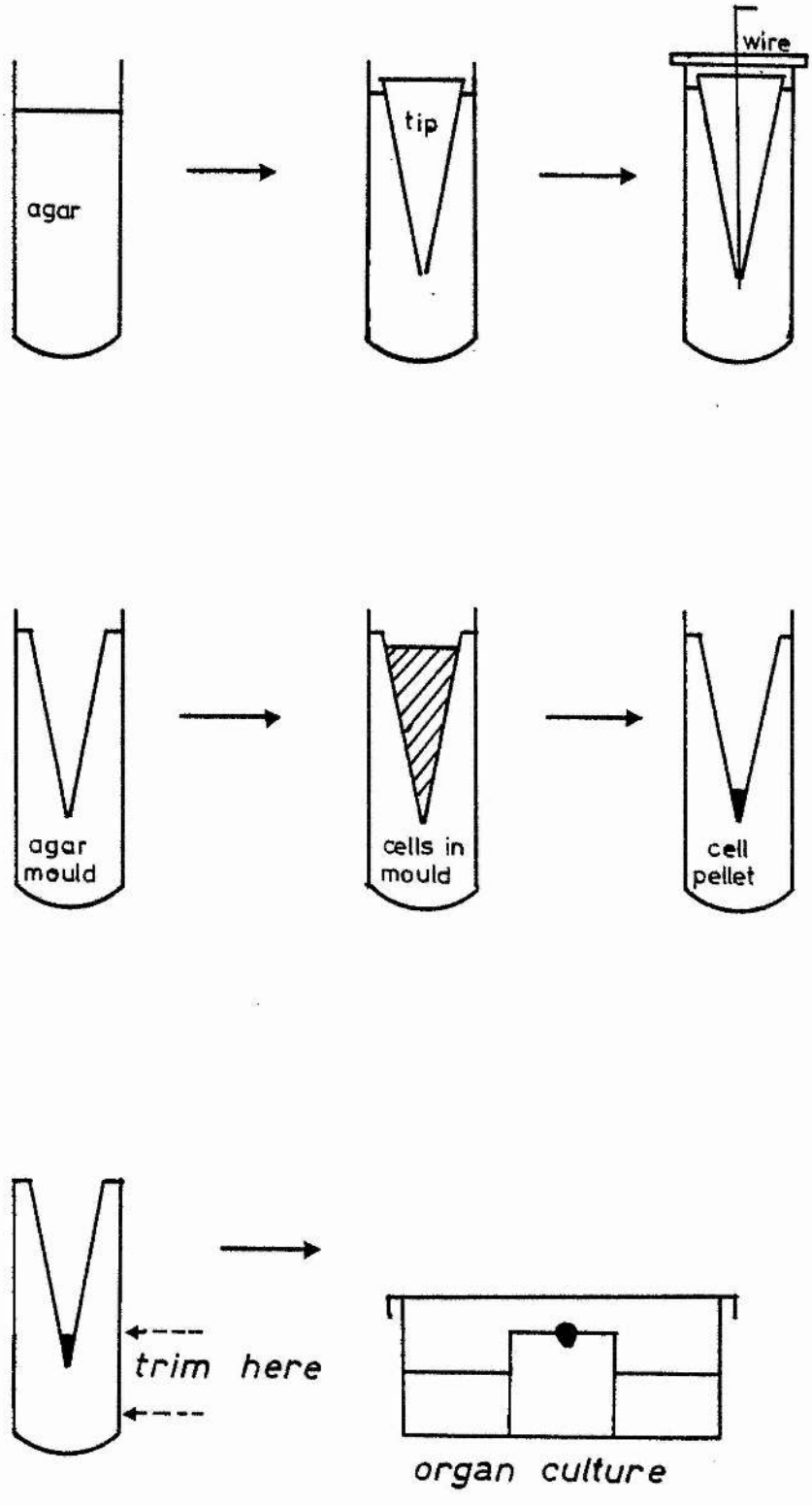
(b) Electron microscopy

The explants were processed as described previously, except the tissue was fixed in glutaraldehyde/paraformaldehyde for 2 h and fixed in osmium tetroxide for 1 h. The tissue was not stained with methylene blue.

6. Method for aggregating cells for organ culture

A 2% solution of agar (Difco) in warm PBS was made up. 3 ml of this was pipetted into 5 ml plastic tissue culture tubes (Falcon). A Finnpipette tip was introduced into the tubes, displacing some of the agar. The caps were replaced, with a small length of wire through them, so that the wire extended through the depth of the Finnpipette tip and protruded slightly into the agar. These procedures were carried out while the agar was still in the liquid state. When it set, the wire was pulled out, letting in air through the orifice at the tip's end and thus breaking the previously air-tight seal around the Finnpippette tip. The tip was then removed, leaving a conical mould in the agar. A cell suspension was then centrifuged in the tube, resulting in an aggregated pellet of cells in the bottom of the agar cone. The supernatant was then removed and the entire agar block removed from the tube by piercing a small hole in the bottom of the plastic tube and blowing out under pressure with a gas line applied to the hole. The agar block containing the pellet of cells was trimmed to a convenient size and placed upright in a petridish containing medium. The pellet of cells was then cultured as in the conventional organ

DIAGRAM 1 ORGAN CULTURE OF CELLS ON AGAR



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culture system, the agar being readily permeable to medium.

C. Assays for immunocompetence/maturation

1. Detection of Thy 1 by immunocytochemistry

The presence of the T cell membrane antigen Thy 1 was looked for on the surface of different cell populations. Both direct and the indirect 'sandwich' technique with specific antiserum was used. Both these methods involved using the enzyme horse-radish peroxidase (HRPO, Sigma) coupled to antibody to detect the antigen.

(a) The direct technique

The IgG fraction of 1 ml of mouse anti-Thy 1 serum (AKR anti-C_3H), a kind gift of Dr. Gani (Searle Research Laboratories) was purified and coupled to horse-radish peroxidase by the following method:-

1.0 ml serum

Dialysed x 3 with 0.01M sodium phosphate ($PO_4^{=}$) buffer pH 6.8

Loaded onto a DEAE cellulose (Sigma) column equilibrated with the same buffer

Eluted sequentially with 0.04M NaCl
0.05M NaCl
0.075M NaCl
0.15 M NaCl

This was necessary as not enough protein came off at lower ionic strengths, as the sample was probably contaminated with other serum proteins.

Absorbance read at $E^{280\ nm}_{10\ mm}$ and protein conc. measured

Dialysed x 3 against 0.125M $PO_4^{=}$ buffer pH 6.0 containing 0.15 M NaCl

Concentrated on a 'Minicon B125', membrane cut off at 50,000 daltons

Centrifuged to remove precipitate

Absorbance read at $E^{280}_{10\ mm}$

2.2 mg of 'IgG' obtained in 0.6 ml, added 0.2 ml $PO_4^{=}$ buffer. Total volume = 0.8 ml

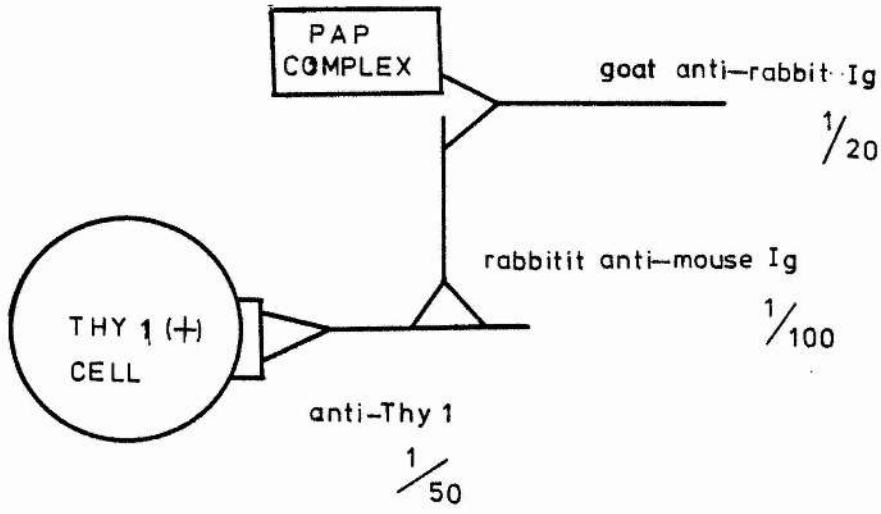
The IgG was coupled to the enzyme by the method of Ternynck and Avrameas (1976 and 1977). The 0.8 ml of protein solution was added to 0.2 ml of a solution of p-benzoquinone (Sigma) of conc. 30 mg/ml (dissolved in ethanol). The reaction mixture was incubated for 1 h at room temperature on a slow stirrer in the dark. This was then loaded onto a Sephadex G.25 (fine) column made up in a 5 ml disposable syringe barrel in saline. The pink coloured fraction was collected and 3 mg of the enzyme HRPO was added. The enzyme was pre-dialysed against saline to remove contaminating salts. A 1/10th of the final volume of 1M Na_2CO_3 buffer pH 9.0 was added and incubated for 15-24 h in the dark at room temperature. The reaction was stopped by adding the new final volume of 1M lysine and incubating in the dark for 2-3 h. The lysine solution was made by dissolving lysine hydrochloride (BDH) in distilled H_2O and adjusting to pH 7.5 by the addition of 1M NaOH. 10 mg/ml of bovine serum albumin (BSA, BDH) was added to help preserve

the preparation, then dialysed against PBS, aliquoted and stored at -20°C.

The reagent prepared by this method was used to detect Thy 1 on cytocentrifugre preparations of cells. Cells were spun on a cytocentrifugre without the presence of serum and fixed in methanol containing 0.2% conc. hydrochloric acid (HCl) to block endogenous peroxidase activity. HRPO conjugated anti-Thy 1 antiserum was applied to the slide for 30 min at a $^{1}/_{5}$ dilution. The slides were washed in tris(hydroxymethylmethylamine, Sigma) buffered saline (TBS) for 30 min and a 0.05 % solution of diaminobenzidiine (DAB, Sigma) was applied to the slides for 5 min, washed in running H_2O for 10 min and finally stained in Mayer's haemalum for 1 min and mounted in styrene.

(b) The indirect method

Cytocentrifuge preparations were made as described above, fixed in acified methanol and treated with a $^{1}/_{50}$ dilution of the anti-Thy 1 antiserum for 30 min. The slides were washed with TBS for 30 min, then treated sequentially with rabbit anti-mouse Ig (1/100 dilution), goat anti-rabbit Ig (1/20 dilution) and finally the peroxidase-anti-peroxidase complex (1/40 dilution). These reagents were supplied by Dako Laboratories. Each antiserum was applied for 30 min with 30 min washes in TBS inbetween. Slides were then stained with DAB and Mayer's haemalum as before.



2. Mitogen stimulation of cells

Lymphoid cells from lymph node, thymus, spleen and bone marrow were cultured in plastic microculture trays (Nunc). Single cell suspensions were made by teasing apart the organs in cold RPMI-Hepes medium. Bone marrow cells were obtained by flushing out the femoral marrow cavity with a syringe containing medium. The plug of marrow obtained was aspirated several times in a pasteur pippette and a single cell suspension was obtained. The cell suspension was washed x 3 in cold RPMI-Hepes in 10 ml disposable plastic tubes. After centrifuging and resuspending, the tubes were allowed to stand on ice for a few minutes to allow the stromal debris to sediment. The supernatant containing the cells was collected. Bone marrow nucleated cells were purified from contaminating erythrocytes by treating centrifuged pelletted cells to osmotic shock by adding a few drops of sterile distilled H_2O. The tubes were immediately filled with medium to restore the isotonicity and allowed to stand for a few minutes on ice. The

lysed erythrocyte debris sedimented and the supernatant containing the resuspended cells were centrifuged and washed. The viability of the cells was determined by a trypan blue dye exclusion test and the cells were resuspended to the required concentration in RPMI 1640 medium buffered with $NaHCO_3$ and supplemented with HIFCS, glutamine and penicillin/streptomycin. These additives were used at the same concentrations as described previously. In some cultures, the medium was additionally buffered with Hepes. 0.1 ml of the cell suspension was added to replicate wells and 0.1 ml of the mitogen PHA (Wellcome) was added to the wells. The final conc. of the cell suspensions or mitogen used depended on the experiments carried out. The trays were incubated at 37°C under a humidified atmosphere of 95% air/5% CO_2 for a total of 72 h. At 60 h, 0.2 μCi of ^{125}Iodo-deoxyuridine (^{125}IUdR, Radiochemical Centre, Amersham) in a volume of 20 μl was added to each well. Cells were harvested at 72 h, with an automated 'Titertek' cell harvester (Flow), the washed cells being deposited on nylon-fibre discs. These were dried at 60°C for 1 h, then placed into plastic vials and processed through an automatic 'Intertechnique' gamma counter. The radioactivity was measured as counts/min and recorded on a tele-type output.

3. Sensitivity to testosterone

A stock solution of testosterone acetate (Sigma) was made up by dissolving 10 mg of the hormone derivative in 1 ml of 70% ethanol and stored at 4°C. 25 μl of the stock solution was added to 5 ml of a thymocyte cell suspension of conc. 2×10^6/ml, mixed and incubated for 10 min at 37°C. 0.5 ml of a 0.5% trypan blue

solution in saline was added and 2 min later, the number of viable cells was counted on an Improved Neubauer haemocytometer. At least a 1000 cells were counted and the % viability determined. To test the effect of the alcohol, used here as a solvent, 20 µl of 70% alcohol was added to 2 ml of a thymocyte suspension (5×10^6/ml) to give a final conc. of 0.7% alcohol.

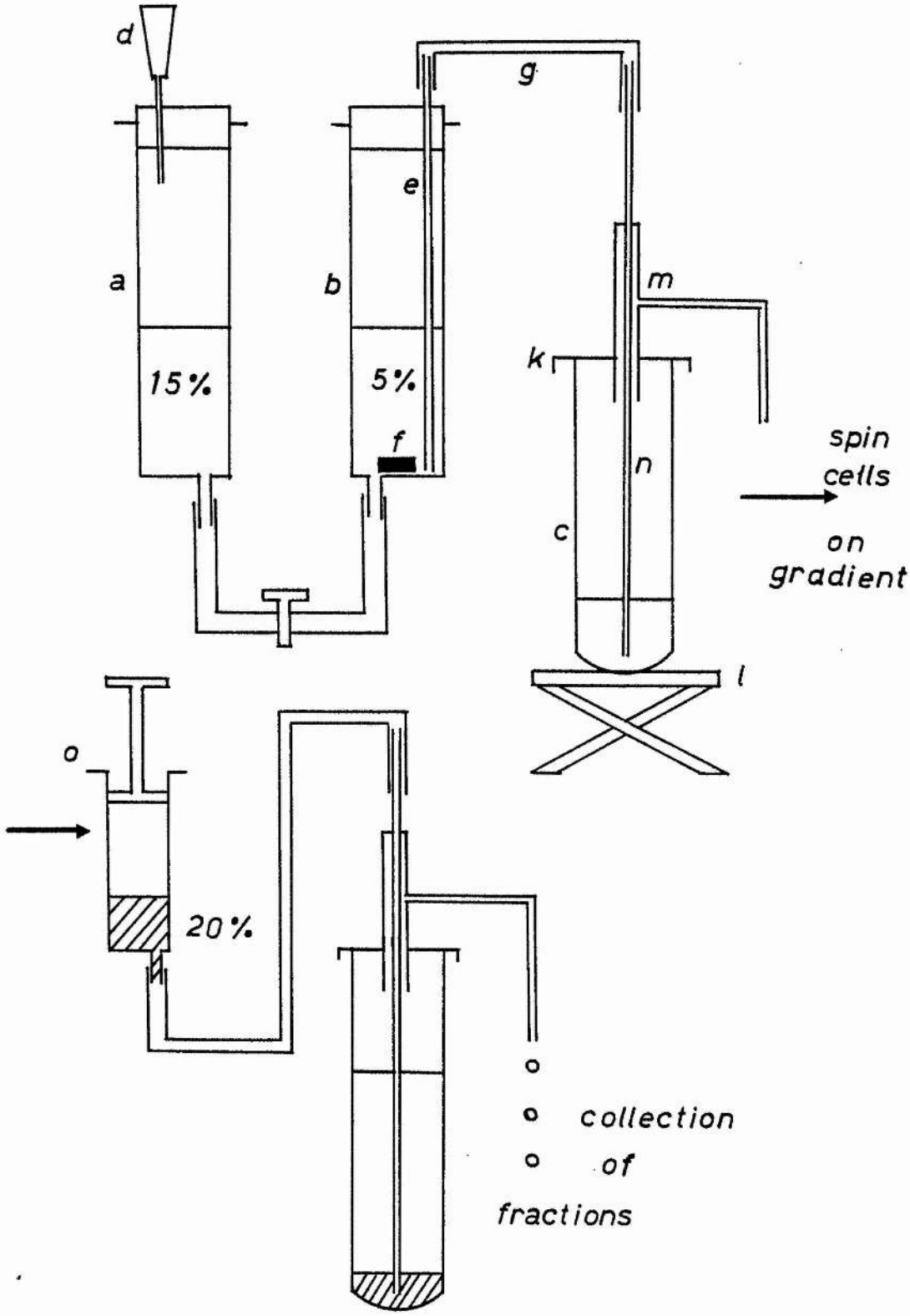
D. Fractionation of bone marrow cells on sucrose density gradients

Linear sucrose density gradients were obtained by mixing solutions of 15% in dist. H_2O and 5% sucrose (BDH) made up in PBS, in a gradient maker constructed as (see diagram):-

The chambers of the gradient maker comprised of two 5 ml disposable syringe barrels (A) and (B), interconnected by a length of silicone rubber tubing (E), clamped in the middle. The chambers were sealed with silicone rubber bungs, bored to accommodate a syringe needle (D) in (A) and a narrow stainless steel tube (E) in (B). Both barrels were clipped to a wooden support which was clamped to a retort-stand. (B) contained a magnetic flea (F) and a stirrer was positioned below it. A 20 ml plastic round bottom tube (C) with a loose-fitting metal cap (K) was supported on an adjustable lab-jack (L) below the level of the gradient maker's chambers. The metal cap was bored through the centre to accommodate a tight-fitting cylindrical metal tube with a side-arm (M). A narrower, loose-fitting tube (N) was fitted through this, which reached the bottom of the plastic tube (C).

7.5 ml of 5% sucrose was put into barrel (B) and the same amount of 15% sucrose into (A). The bungs were replaced and a silicone rubber tube (G) was connected between the tops of tubes

DIAGRAM 2 DENSITY GRADIENT MAKER AND SEPARATION OF CELLS



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(E) and (N). The clamp between (A) and (B) was removed and a small amount of pressure was introduced into (A) by fitting a separate empty syringe onto needle (D) and squirting gently to prime the flow. The magnetic stirrer was started and the first few drops reaching (C) were pure 5% sucrose. The lab-jack was adjusted to maintain a constant difference in height between the fluid levels in the chambers and in (C), so that a constant 1 ml/min was delivered in (C). The lower density sucrose was displaced upwards in (C) by the progressively denser solution. The tube (G) was clamped before all the fluid in (B) was drained, in order to avoid air bubbles. The cap (K) plus attachments was carefully removed to avoid disturbing the gradient.

1 ml of the cell suspension was layered on top of the gradient and the tube (C) was centrifuged at 100 G for 5 minutes. The cap (K) and its attachments were replaced and a 20 ml syringe (O) containing 20% sucrose, dyed with toluidine blue was attached to the tube (G), from which the clip was removed and the denser sucrose displaced the gradient upwards and out of the side-arm on (M). The syringe (O) containing the reservoir of denser sucrose was positioned so as to displace the gradient at a rate of approx. 1 ml/min, the fractions being collected in 1 ml aliquots into 5 ml plastic tubes. The fractions were washed x 3 in cold RPMI-Hepes containing serum and the cells were either cultured or examined morphologically to determine the distribution of the nucleated cells in the various fractions.

E. Cytokinetics of thymocytes

1. The thymus and spleen during pregnancy

(a) Time study

CBA mice were set up in mating colonies of 3 females to 1 male as described previously. The animals were 8-10 weeks of age and the males and females of each colony were litter-mates. At 5, 10, 15 and 20 days of gestation, they were sacrificed by ether asphyxia and their thymuses and spleens removed and weighed. One thymic lobe was processed for histology and the other was teased apart in cold PBS and a single cell suspension made by homogenising the tissue. 40 μl of the cell suspension was diluted in 20 ml of Isoton solution and the cellularity of the organ determined by replicate cell counts of the suspension in a 'Coulter' counter.

(b) ^{59}Fe uptake during pregnancy

A separate batch of female CBA mice were mated as described above. On day 14 of gestation, 1 μCi of ^{59}ferric chloride ($^{59}FeCl_3$, Radiochemical Centre, Amersham) was injected i/v through a tail vein; 24 h later the mice were killed, the spleens and femurs removed and the radioactivity measured in a gamma counter.

2. Mitotic arrest by vincristine

Female non-pregnant CDI mice (Charles River) weighing approx. 25 g were injected i/v with vincristine sulphate (Eli-Lilly) at doses of 1-5 mg/Kg body weight. The drug is prepared from an alkaloid and is a stathmokinetic agent. It inhibits spindle formation during mitosis and thus enables detection of dividing cells by the clumped appearance of their chromatin. 2 h later, the

animals were killed and the thymuses removed and processed for histology. They were bisected down the middle of the longitudinal axis and blocked with the cut surface exposed. This enabled a true cross-section to be examined and the distribution of metaphase nuclei across the tissue to be determined. The sections were viewed under an X100 oil immersion lens of a Leitz microscope. The number of metaphases in adjacent parallel fields or areas of a grid, across the section was counted.

CHAPTER 1

Thymus monolayer cultures

INTRODUCTION

It is now recognised that the cellular differentiation events within the thymus involve the complex interaction of several distinct populations of cells, which result in the generation of subsets of T lymphocytes (reviewed by Cantor and Weissman, 1976). Of fundamental importance to this process is the role of the thymic non-lymphoid cells, in particular the reticuloepithelial and stromal elements. The vestigial thymic rudiment of the nude mouse has a congenital dysfunction of these cells, which fails to support lymphopoiesis (Wortis et al, 1971b and Owen et al, 1975). The humoral secretions of the thymus and their inductive effects have been extensively investigated (reviewed by Bach and Carnaud, 1976). A primary interaction between pre-T cells and the reticuloepithelial cells, or at least a humoral effect exerted by their secretions within the thymic environment is an obligatory pre-requisite in lymphopoiesis.

Several investigators have approached the problem of thymic lymphopoiesis by investigating the effect of co-culture with monolayers of thymic epithelium on purified haemopoietic cells, or incubation in conditioned medium from such cultures. The literature on the subject is as confusing as is diverse, and is further complicated by the lack of standardised assays to assess lymphoid differentiation. Epithelial monolayers have been derived from the thymic tissue of mice, rats, man and monkey; species differences do not seem to limit their inductive effects on xenogeneic target cells. Such target cells have included spleen cells from athymic and thymus-deprived

mice, thymocytes and bone marrow cells. Differentiation was assessed by the proliferative response to phytomitogens, response in the MLC, helper activity and the E-rosette assay or its sensitivity to azathioprine. These properties, characteristic of mature T lymphocytes have been induced with varying degrees of success. In most of these investigations, thymic epithelium was prepared by explanting small fragments of intact or dissociated thymic tissue into culture flasks and subsequently culturing the adherent cells. Epithelial cells were believed to grow out from the sites of adherence, whilst lymphocytic cells were lost with medium changes. Although it was tacitly assumed by most investigators that the cultured cells were epithelial, the presence of junctional complexes or other epithelial cell criteria were not demonstrated by most.

During the course of this study, evidence from other laboratories demonstrated that the majority of cultured cells thought to be murine thymic epithelium were in fact macrophages. Morphological and functional evidence in support of this contention has been shown. In addition, the histochemistry and radiosensitivity of these cultured cells were found to be characteristic of macrophages rather than epithelium (Jordan et al,1979a; Jordan and Crouse, 1979a) and J.G. Sharp, personal communication). A similar but brief investigation to characterise the cells cultured in this study was undertaken. Following administration of the cytotoxic drug mustine hydrochloride (nitrogen mustard, HN_2) *in vivo*, there is an asynchronous restoration of marrow cellularity, thus leading to a relative enrichment of the haemopoietic stem cell compartment between 2-4 days post-treatment (Sharp and Thomas, 1971 and Riches et al, 1976). The expression of the Thy 1 differentiation

antigen on haemopoietic cells from normal and HN_2 treated mice after co-culture with thymic monolayers was investigated. The lympholytic effect of adrenal corticosteroids are well recognised and their effect is most significant on the thymus. Loss of sensitivity to cortisone is known to accompany thymocyte maturation in vivo. Gonadectomy in rodents is known to result in an increase in weight of the lymphoid organs, including the thymus (Castro, 1974). It is not known whether sex-steroids primarily affect immature thymocytes as cortisone does. In this study, the effect of testosterone on thymocytes in vitro was investigated after co-culture with thymus monolayers and incubation with their supernatants. The polyclonal reactivity to phytomitogens is known to be a characteristic of mature thymocytes. The possible inductive effect of thymic monolayer cells and their supernatants on the PHA responsiveness of thymocytes was investigated.

RESULTS

Growth and Morphology

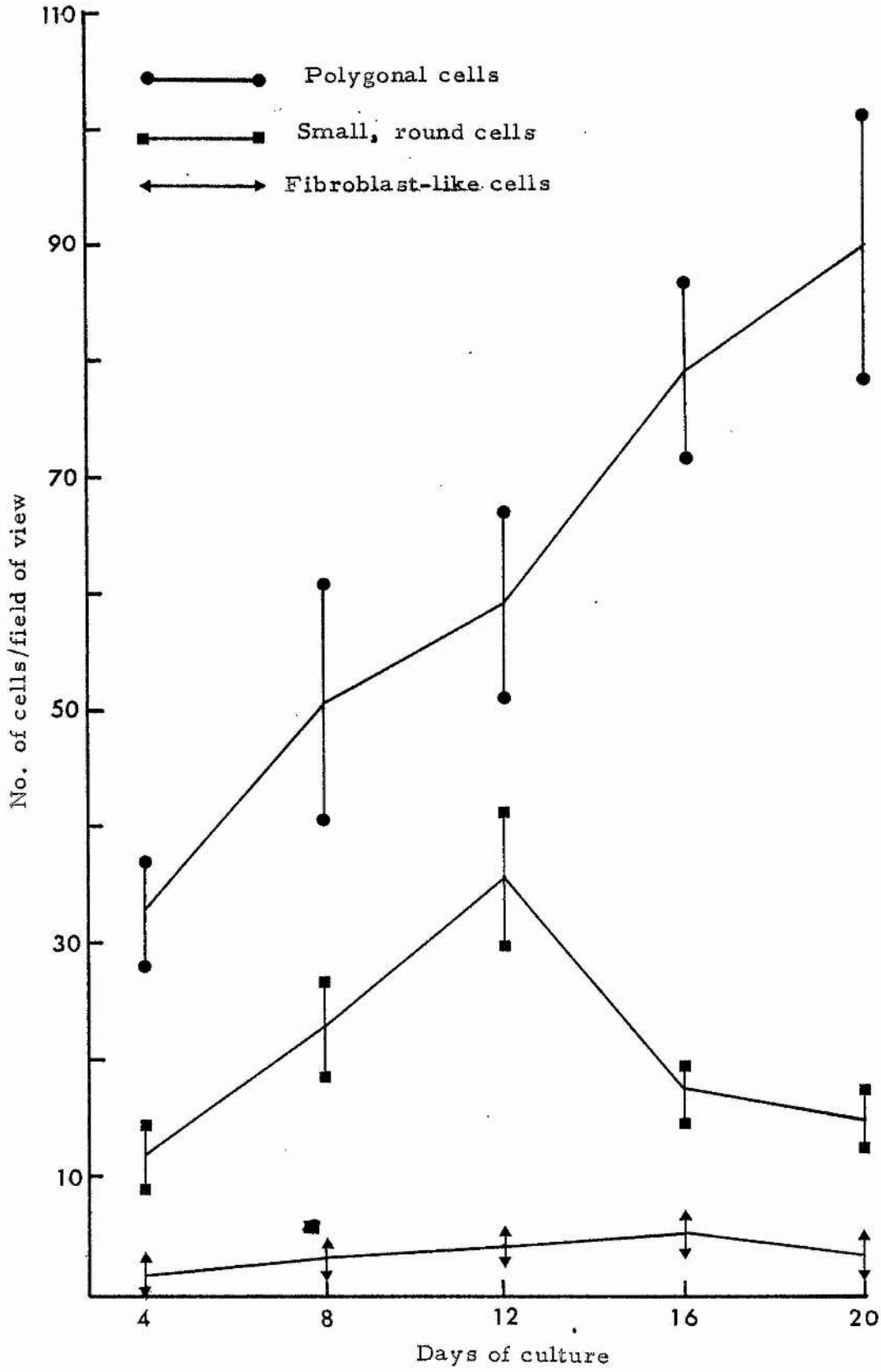
Explants of thymic tissue were maintained in culture flasks as described in the Methodology. Radial outgrowths of non-lymphoid cells from the explants were seen after 24 hours of culture, which adhered to the substrate. The non-adherent thymocytes in the explants were lost with successive medium changes by the first week of culture. The stromal cells reached confluence in approx. 1 month in nearly all cases. Morphologically, the cultured cells could be broadly divided into three types:-

(1) Small, round cells with refractile nuclei, which had the appearance of fried eggs, these were abundant in the early stages of culture.

(2) Small, dendritic cells with short cytoplasmic processes that resembled fibroblasts.

(3) Polygonal cells of variable size with oval, eccentric nuclei which were sometimes binucleate. In culture, their cytoplasm was flattened, spread over a large area, contained vacuoles and was often granulated. They were often seen to be joined by cytoplasmic bridges. Later cultures were composed of predominantly large, spread cells with extended cytoplasmic processes, the small fibroblast-like cells were very few in number throughout the culture. The three main types of cells at randomly chosen locations in culture flasks were scored at intervals of 4 days up to 3 weeks of culture. The number of small, round cells decreased after approx. 2 weeks, whereas the polygonal cells became more numerous throughout the culture period till confluence was reached (Fig. 1).

Fig. (1) Growth rate of cells in monolayer cultures



This suggested that the cells could morphologically transform during culture from the small, round appearance to the larger polygonal shape, the polygonal cells increased in size by spreading out on the substrate.

The cells characteristic of an early culture are seen in Plate (1) which was obtained from a 1 week old culture. The cultured cells were often binucleated with prominent, sometimes multiple, nucleoli. Plate (2) was obtained from a 1 month old culture and is seen to be comprised of essentially large, polygonal cells. For comparison, a photomicrograph of human foetal thymus similarly cultured, is included (Plate 3). The morphology of the cultured cells from both species is similar. In the older cultures, the cells did not appear to exhibit contact inhibition as they frequently grew over and under adjacent cells. They were occasionally seen to pile up on each other to form a ridge-like structure, surrounding a large clear space or "vacuole" in the monolayer. These "vacuoles" were occupied by a few cells growing at a low density, unlike the remainder of the confluent monolayer (Plate 4). A cytocentrifuge preparation of cells trypsinised from a confluent monolayer approx. 1 month old is seen in Plate (5). Unlike the morphological heterogeneity seen in the cultures, the cells appear uniform; their oval, eccentric nuclei appears similar to the large polygonal cells seen in culture. Their cytoplasm appears foamy and highly vacuolated. In these preparations, the cells always appeared juxtaposed to each other, assuming irregular shapes so as to form a continuous carpet of cells. During the trypsinisation process, the cytoplasm of the large polygonal cells

Plate (1) Mouse thymus, 1 week in culture.

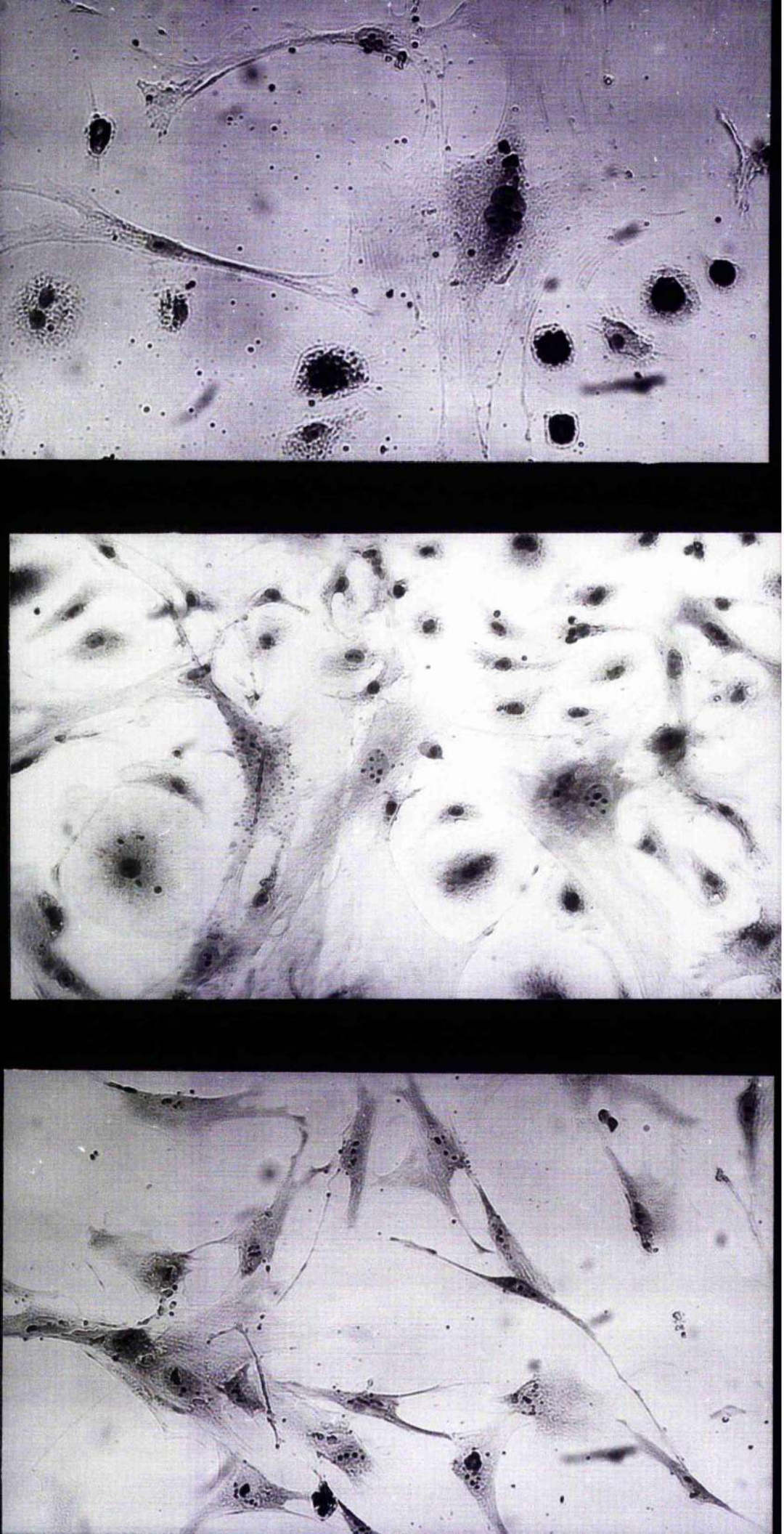
Jenner and Giemsa (J and G) x 480

Plate (2) Mouse thymus, near-confluent after 1 month in culture

J and G x 480

Plate (3) Human foetal thymus, 1 week in culture

J and G x 480



were seen to be peeled back at their edges first and became ruffled in appearance. This observation would be consistent with the notion of them shrinking to the size of the smaller cells seen in the cytocentrifuge preparations.

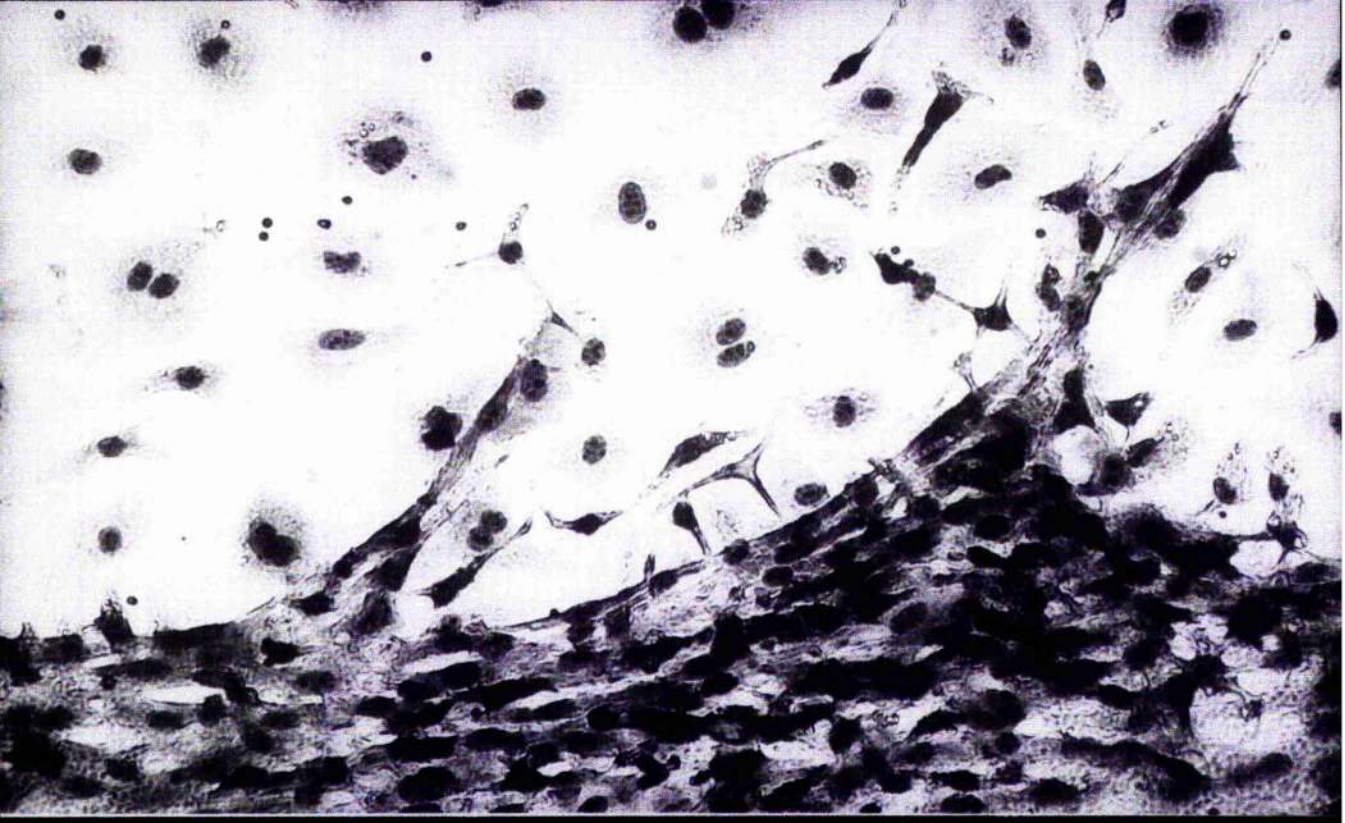
Plates (6) and (7) are electronmicrographs of a trypsinised pelleted cell. The cell has an irregular surface, mitochondria are sparse and there is a prominent golgi with numerous vesicles. The nucleus has a denser peripheral heterochromatin and unlike the smooth oval nuclei seen in cytocentrifuge preparations, appears to be indented and bean-shaped. In order to examine the cells as seen in culture at the ultrastructural level, the monolayers were fixed and partly processed in situ. Plate (8) is a lower power picture of a cell in culture alongside the cytoplasmic process of another. The cell is grossly irregular and the cytoplasm is poorly defined. There are a large number of vacuoles and intensely osmiophilic structures, typical of the changes seen in cells after some days in culture. There are few mitochondria and free ribosomes are abundant, the cell exhibits no polarity of organisation. The vacuole-like structures could represent secondary lysosomes. Plate (9) is a higher power picture of another cell from the same culture. There is a well-defined golgi complex, numerous mitochondria, free ribosomes and possibly a centriole. The processes of cells in culture were often seen apposed to each other and the possible presence of junctional complexes was investigated. Plate (10) shows 3 cells in close proximity to each other. Although an extensive search was not carried out, nothing resembling desmosomes was ever seen. The presence of vesicles was a

Plate (4) "Vacuole" formation in monolayer of 5 week old culture, surrounded by ridge of multilayered cells.

J and G x 480

Plate (5) Cytospin preparation of trypsinised cells from a confluent monolayer, 1 month old.

J and G x 1200



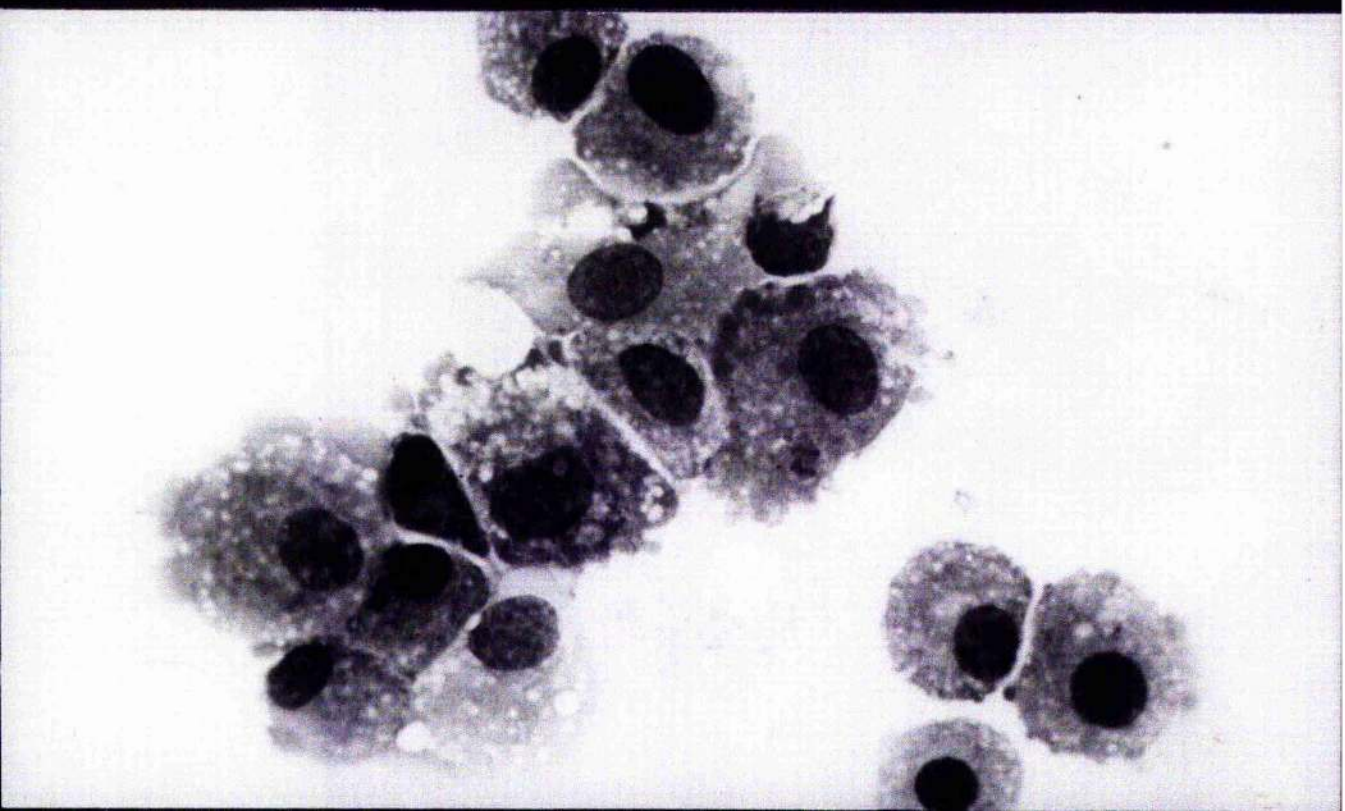


Plate (6) Electronmicrograph of trypsinised pelleted cell.

An irregular surface, sparse mitochondria and prominent golgi with vesicular cytoplasm are evident. The bean-shaped nucleus has denser peripheral heterochromatin.

x 1100

Plate (7) Higher magnification of above.

x 20, 300

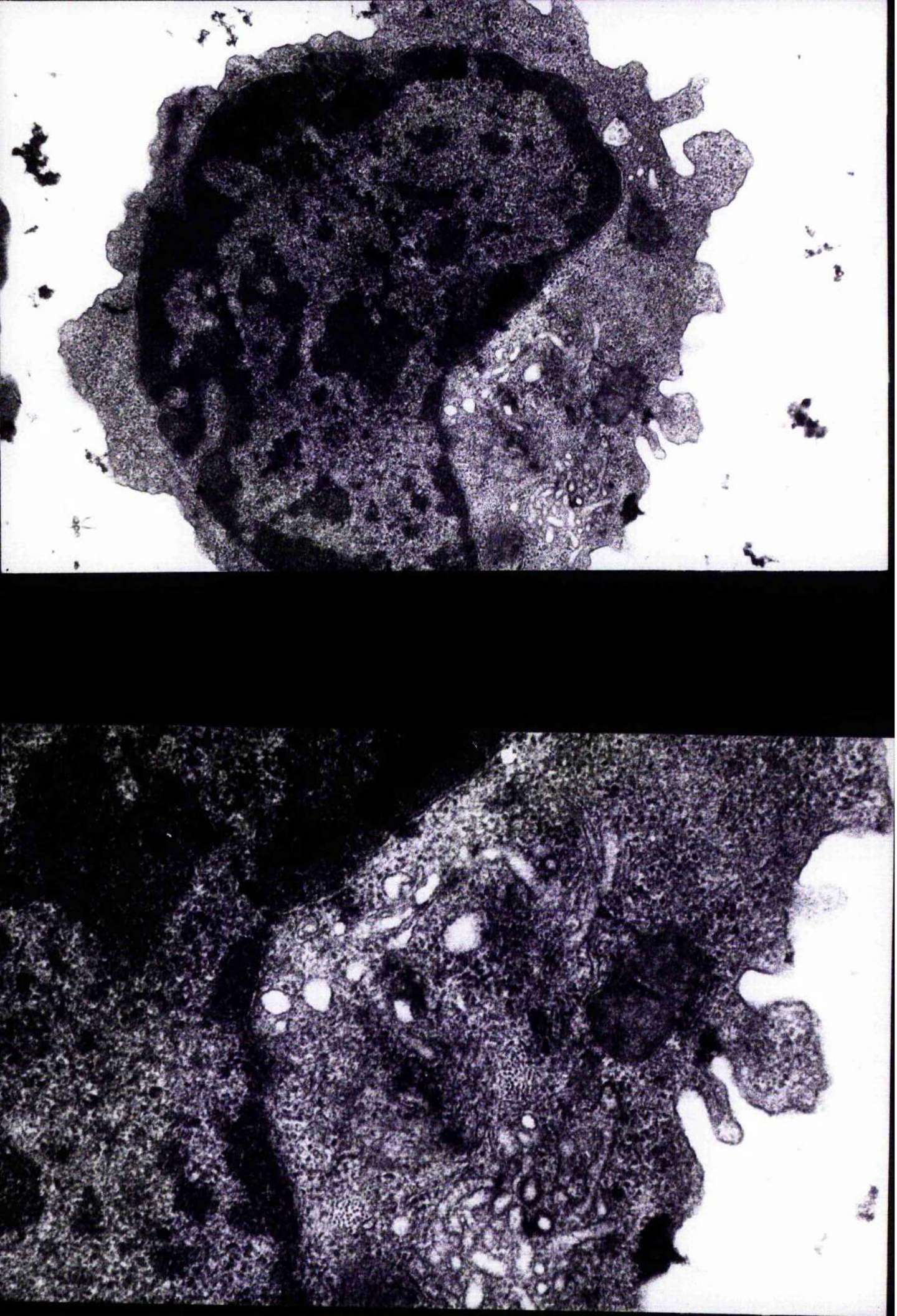
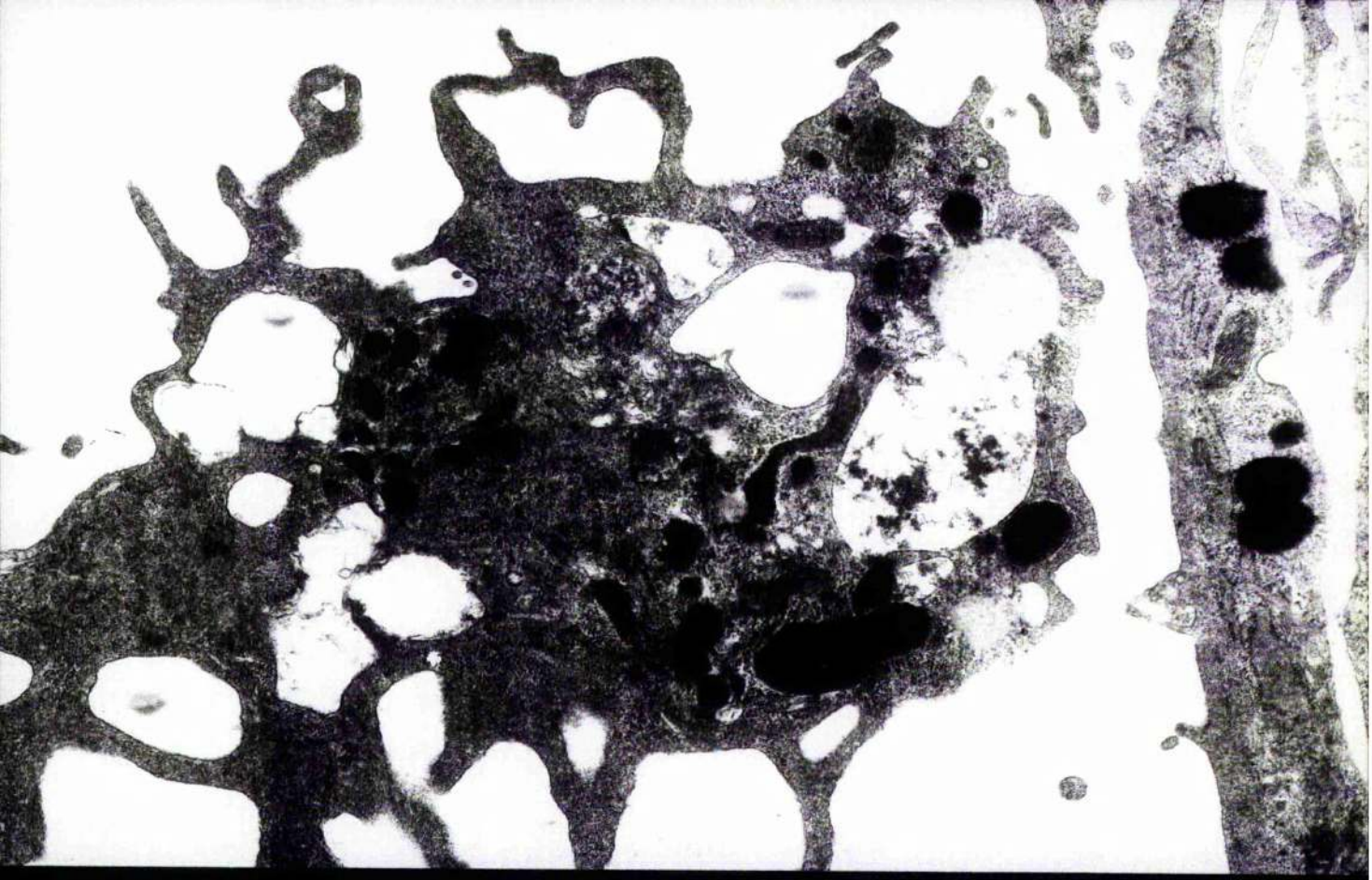


Plate (8) A grossly irregular cell in culture seen beside the cytoplasmic process of another. The cytoplasmic organisation is poorly defined. Fixed and stained in situ.

x 6000

Plate (9) Higher power electronmicrograph of another area of monolayer from the same culture as above. Cytoplasmic organelles are clearly discernible.

x 26,000



prominent feature in these cultured cells. Plate (11) shows a cell with sac-like structures extruding material into large membrane-bound vesicles. The lower vesicle is in close association with what appears to be a swollen golgi complex or possibly smooth endoplasmic reticulum. Cells in monolayer culture were usually found to deteriorate after approx. 6 weeks in culture. Plate (12) is obtained from such a senescent culture. There is an abundance of osmiophilic (lipid) material and vesicles containing membraneous material.

Characterisation of thymus monolayer cells

Histochemical and functional assays were performed on the monolayer cells in situ in the culture flasks. The cells were stained for the presence of cytoplasmic non-specific esterases and assayed for their ability to phagocytose unsensitised heat-killed yeast cells in vitro.

The majority of cells in culture were positive for both tests. They contained non-specific esterases and were darkly stained, a few polygonal cells were very lightly stained and it is difficult to categorise them. The same esterase positive cells were found to be actively phagocytic. A very few isolated cells were occasionally seen that were negative in both assays and were weakly PAS positive, they were too few in number to be counted. Plate (13) shows a monolayer derived from a young adult mouse which has phagocytosed the yeast cells, the darkly stained areas detect the enzyme esterase which appears dark brown under the microscope. Monolayers were also established from trypsinised 16 day foetal

Plate (10) Cytoplasmic processes of three cultured cells in close apposition to each other.

x 58,500

Plate (11) Cultured monolayer cell with typical vesicular cytoplasm.

x 19000

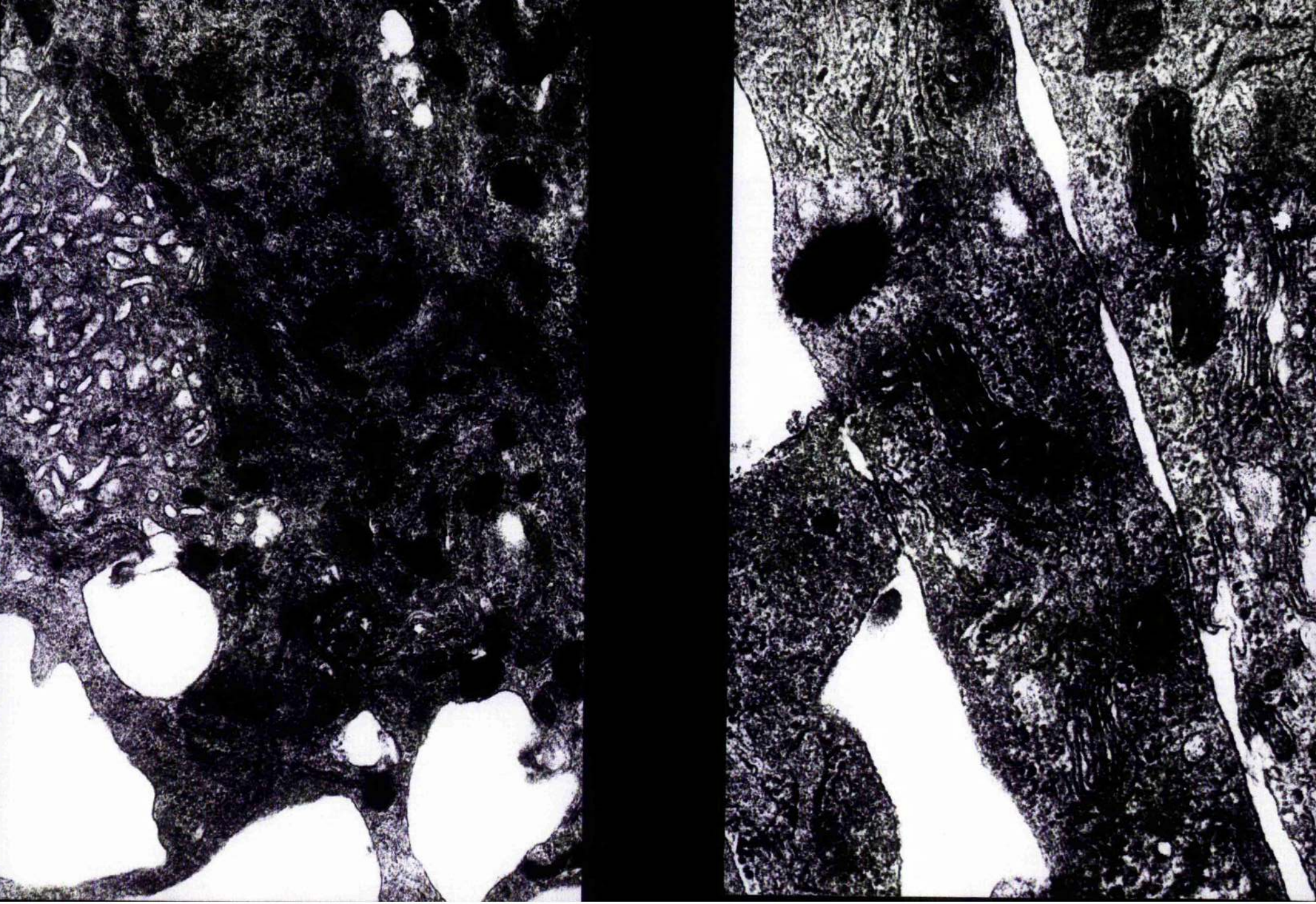
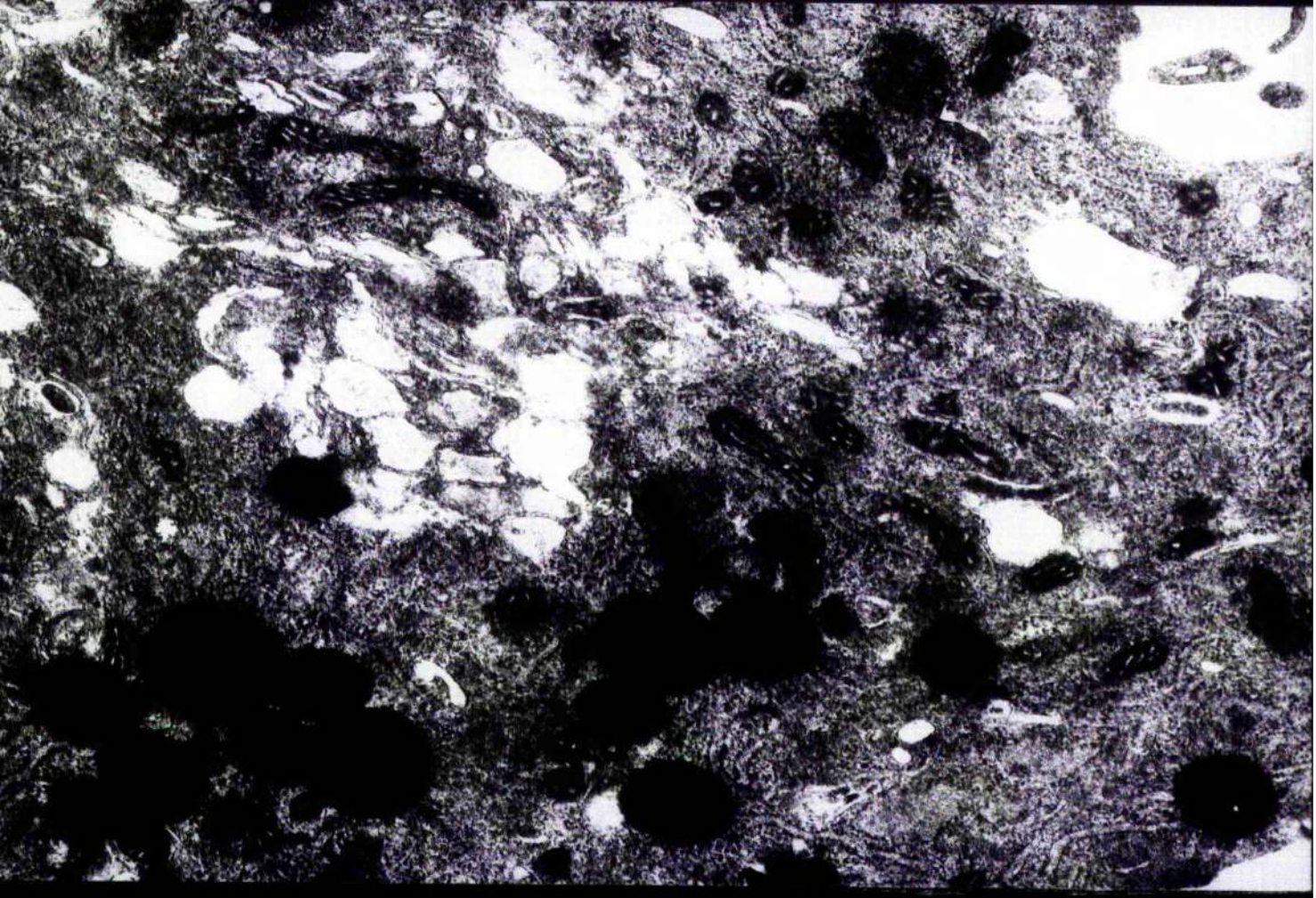


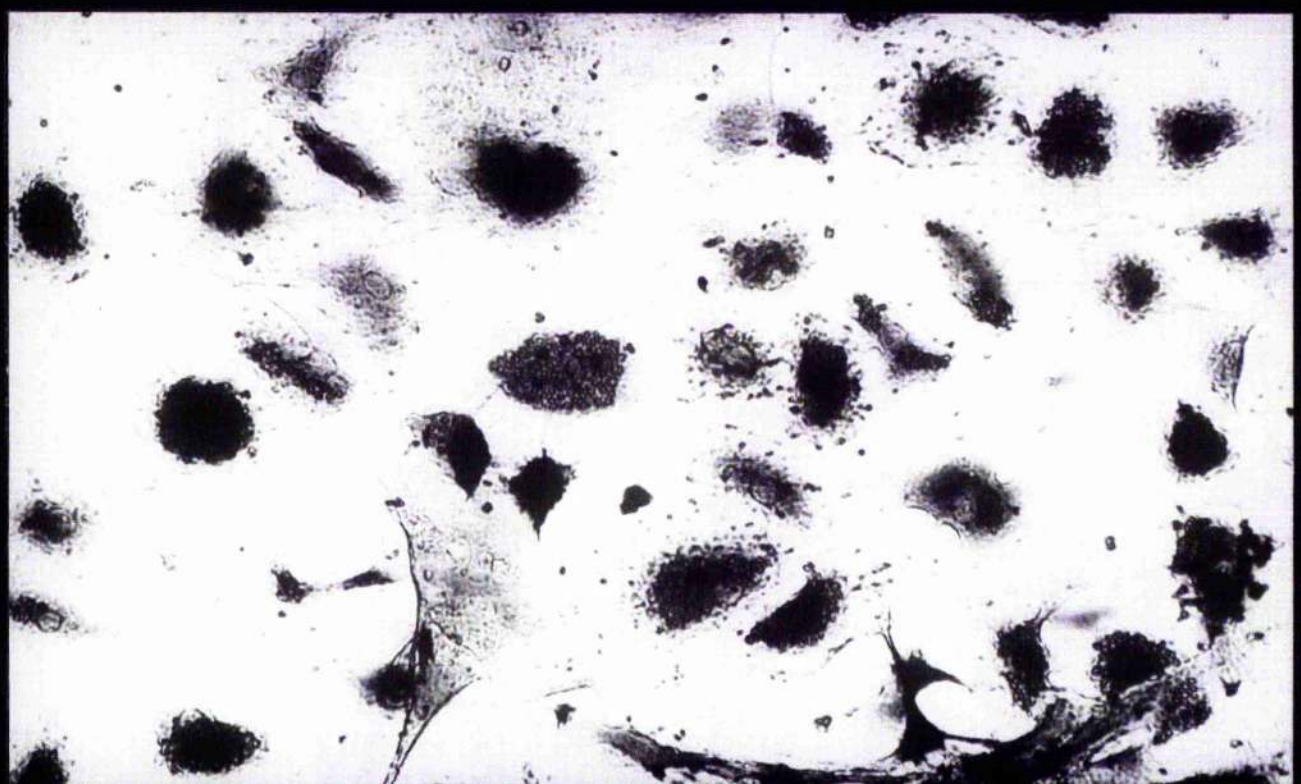
Plate (12) Degenerating monolayer cell from a senescent 7 week old culture containing osmiophilic inclusions.

x 236000

Plate (13) Esterase positive, phagocytic cells from thymus of young adult mouse. Counterstained with Haematoxylin

x 480





mouse thymus, these cells were also found to be positive in the macrophage assays. Plate (14) shows cells from an early culture that have been similarly treated. Mouse peritoneal exudates, being rich in macrophages, were similarly cultured for comparison. The cultures generally took longer to reach confluence, at which stage they appeared very similar to cultured thymus cells. Plate (15) shows such a culture, six weeks old. As expected, these cells were positive for non-specific esterase and phagocytosed yeast cells (Plate 16).

The possible presence of Fc receptors (FcR) on cultured thymus stromal cells was investigated in situ, using sensitised ox erythrocytes. Occasionally, cells with erythrocytes on their surfaces were seen as in Plate (17). However, as their numbers were relatively low (less than 20%) and they occurred inconsistently in cultures, it is doubtful whether they are true rosettes. When peritoneal exudate cells were similarly treated, about half of the cells had clusters of 5 or more erythrocytes around them, as seen in Plate (18); these were considered to resemble more typical rosettes.

As the cultured thymic stromal cells were largely found to be macrophages, their ultrastructure was compared to their in vivo counterpart. A high-power electronmicrograph of the subcapsular region of a 15 day foetal mouse thymus is shown in Plate (19). It shows part of a thymic macrophage whose cytoplasm contains many lysosomes and membrane-bound vesicles. The very dark regular structures are probably residual bodies containing lipid

Plate (14) Esterase positive, phagocytic cells from thymus of foetal mouse at 16 days of gestation.
Counter stained with Haematoxylin
x 480

Plate (15) Monolayer of cells from mouse peritoneal exudates (PEC), 6 weeks in culture
J and G, x 480

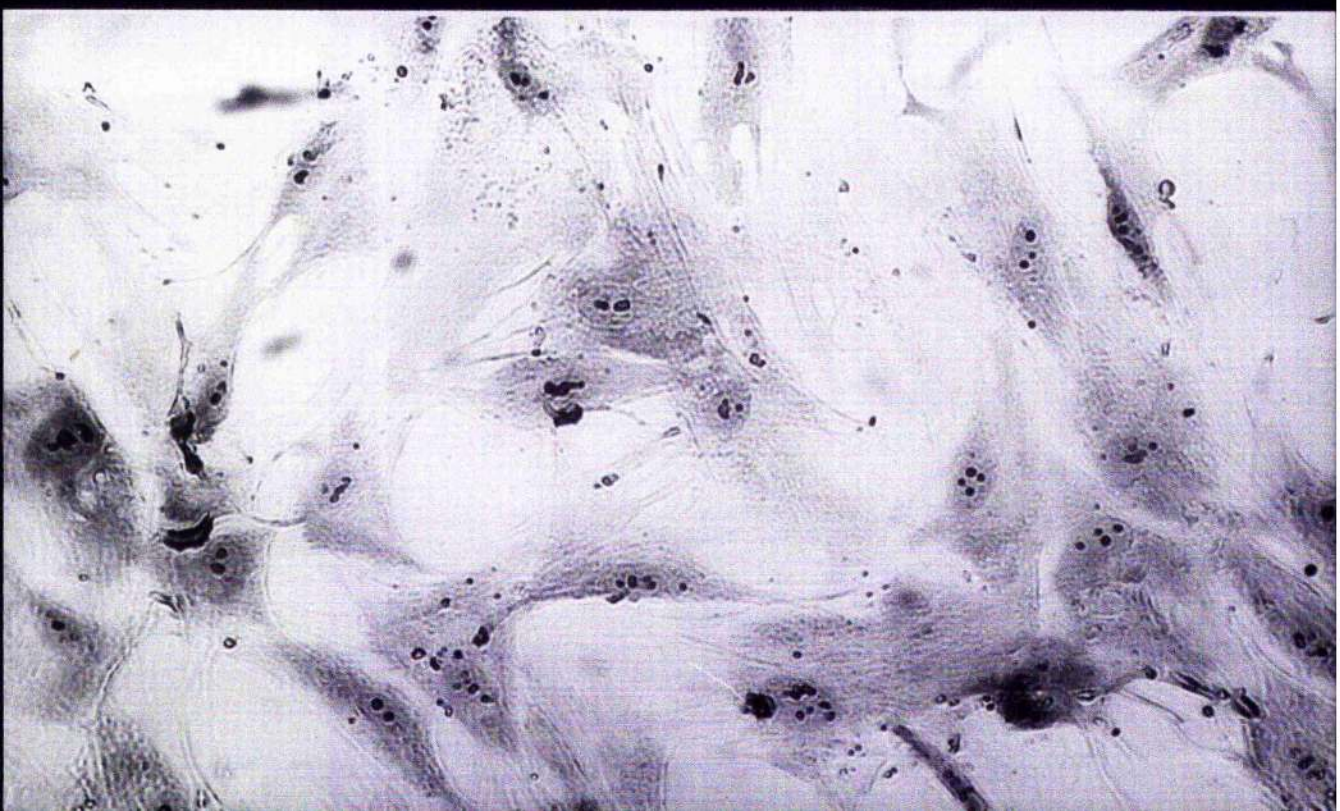
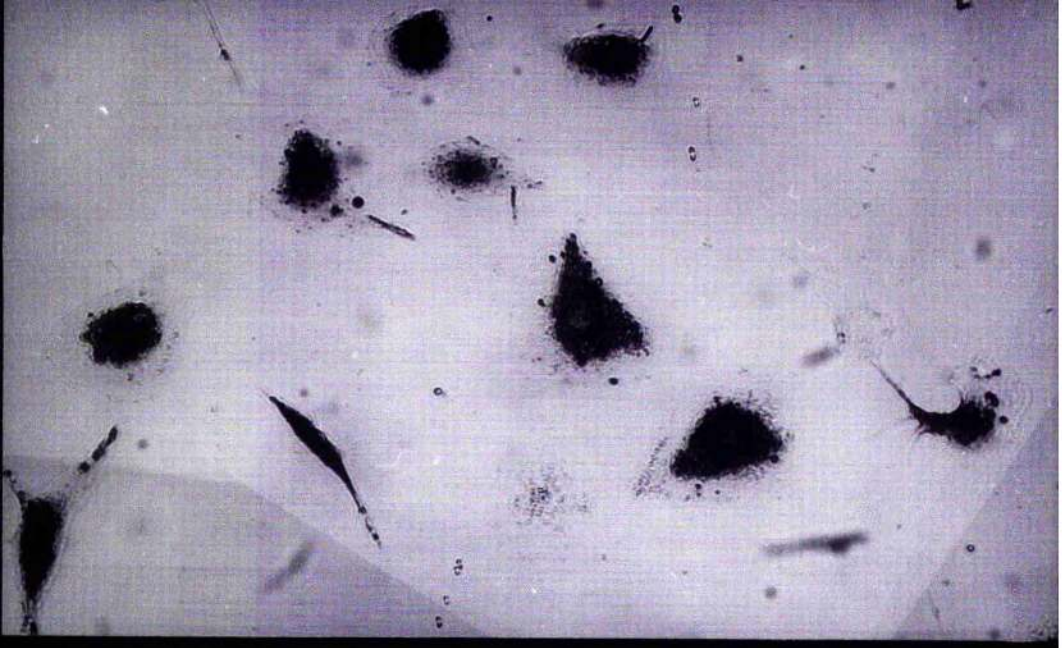
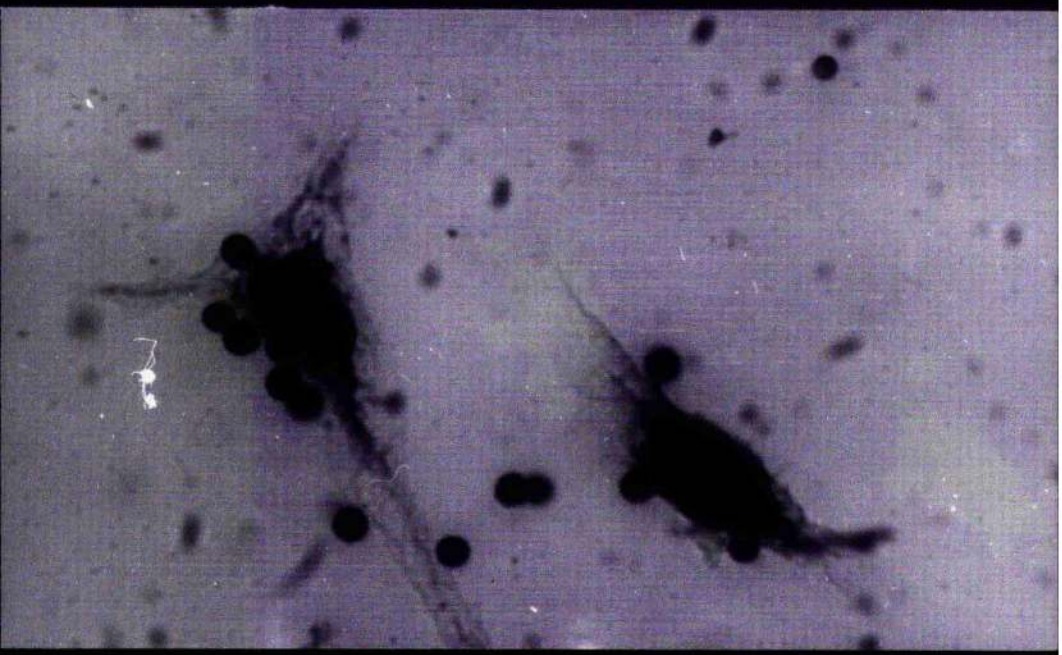


Plate (16) Mouse PEC showing presence of cytoplasmic esterase and phagocytosis of yeast cells.
Counterstained with Haematoxylin
x 480

Plate (17) Thymus cells in early culture treated with sensitised ox erythrocytes to detect Fc receptors (FcR).
J and G x 1200

Plate (18) PEC cells similarly treated to above.
J and G x 1200





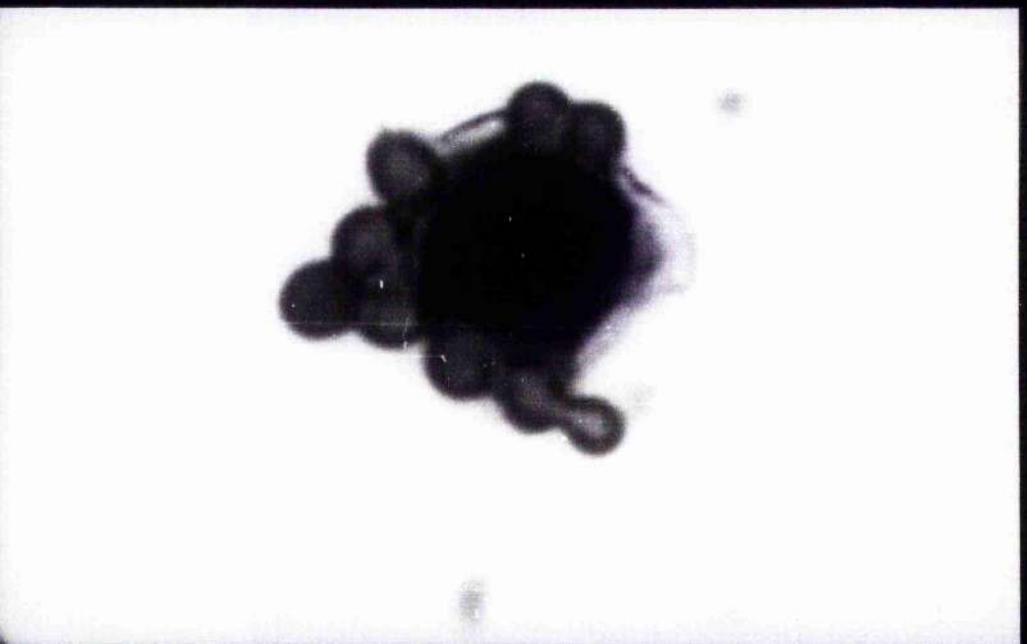
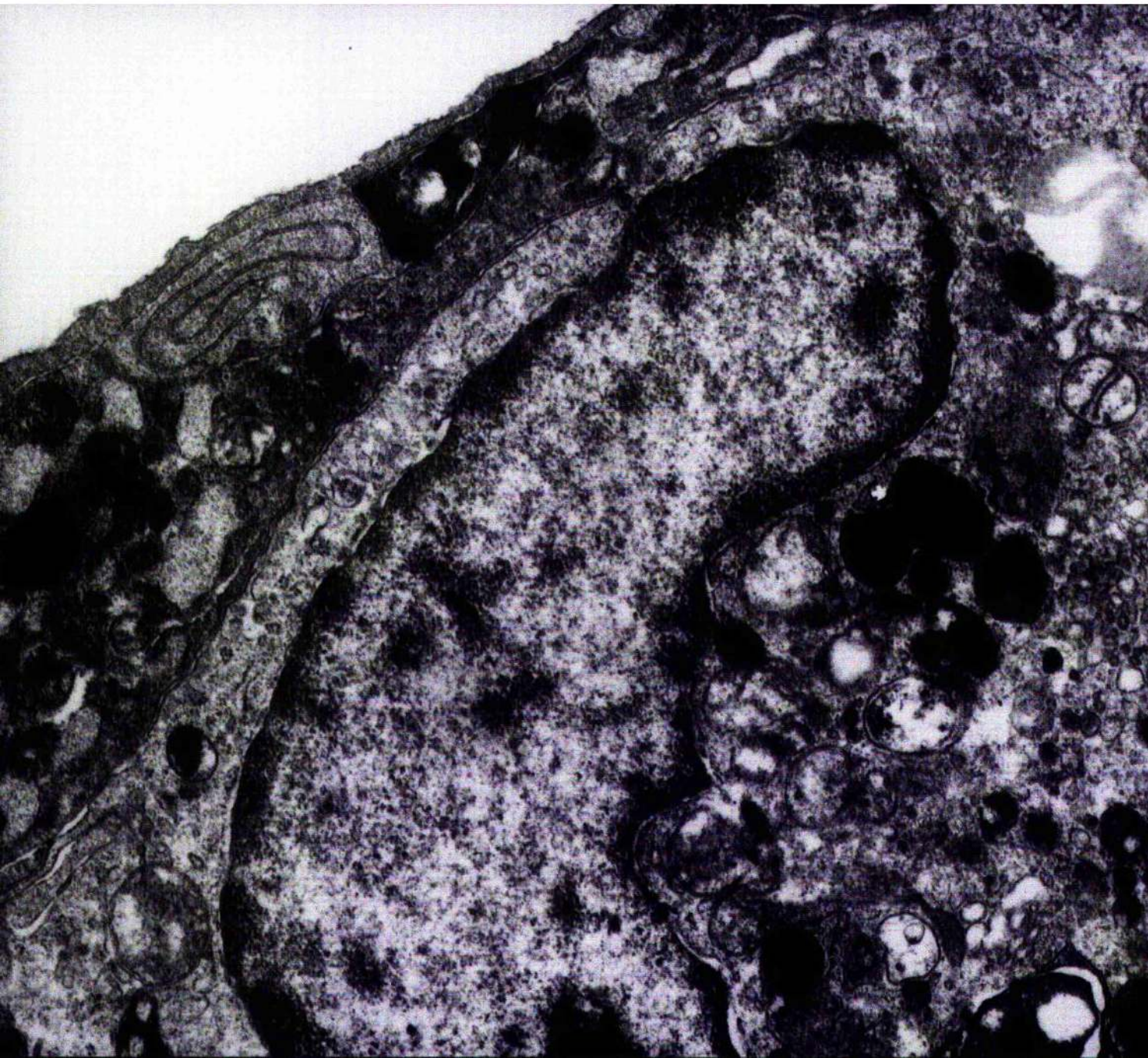


Plate (19) Electronmicrograph of a thymic macrophage *in vivo*, from a 15 day mouse embryo. Lysosomes and membraneous vesicles, together with lipid inclusions are in abundance. The nucleus is elongated, with denser peripheral heterochromatin.

x 16500



after fusion with primary lysosomes. There is a thin rim of peripheral dense heterochromatin in the elongated nucleus. An abundance of free ribosomes and what could be the tubules of a golgi complex cut in transverse section are seen.

Inductive properties of thymus monolayer cells

(1) PHA responsiveness

(A) Dose study

An initial study of the proliferative response of lymph node cells upon mitogenic stimulation with PHA between 1-8 µg/ml and at a cell conc. of 2×10^5 cells/well was undertaken. Fig. (2) shows that maximal stimulation was achieved between 3-6 µg/ml of PHA. The results represent the mean counts per minute (cpm), corrected for isotope decay and background. In the subsequent experimental protocols, it was decided to use the mitogen at 4 µg/ml.

(B) Effect of conditioned medium from thymic monlayers

The PHA response of thymocytes after 24 hours of incubation with conditioned medium (CM) from monolayers of thymus stromal cells was investigated. The CM was obtained after 72 hours of culture unless otherwise stated. The CM from peritoneal exudate cell (PEC) monolayers were also tested as controls. Figs. (3) and (4) show the data from two separate experiments, expressed as cpm. The stimulation indices are shown within the boxes. Supernatants from thymus monolayer cells induced greater stimulation than PEC monolayers in both experiments. Lymph node cells were also cultured under identical conditions in this study, to verify that the culture conditions permitted adequate cell

Fig. (2) PHA response of lymph node cells:

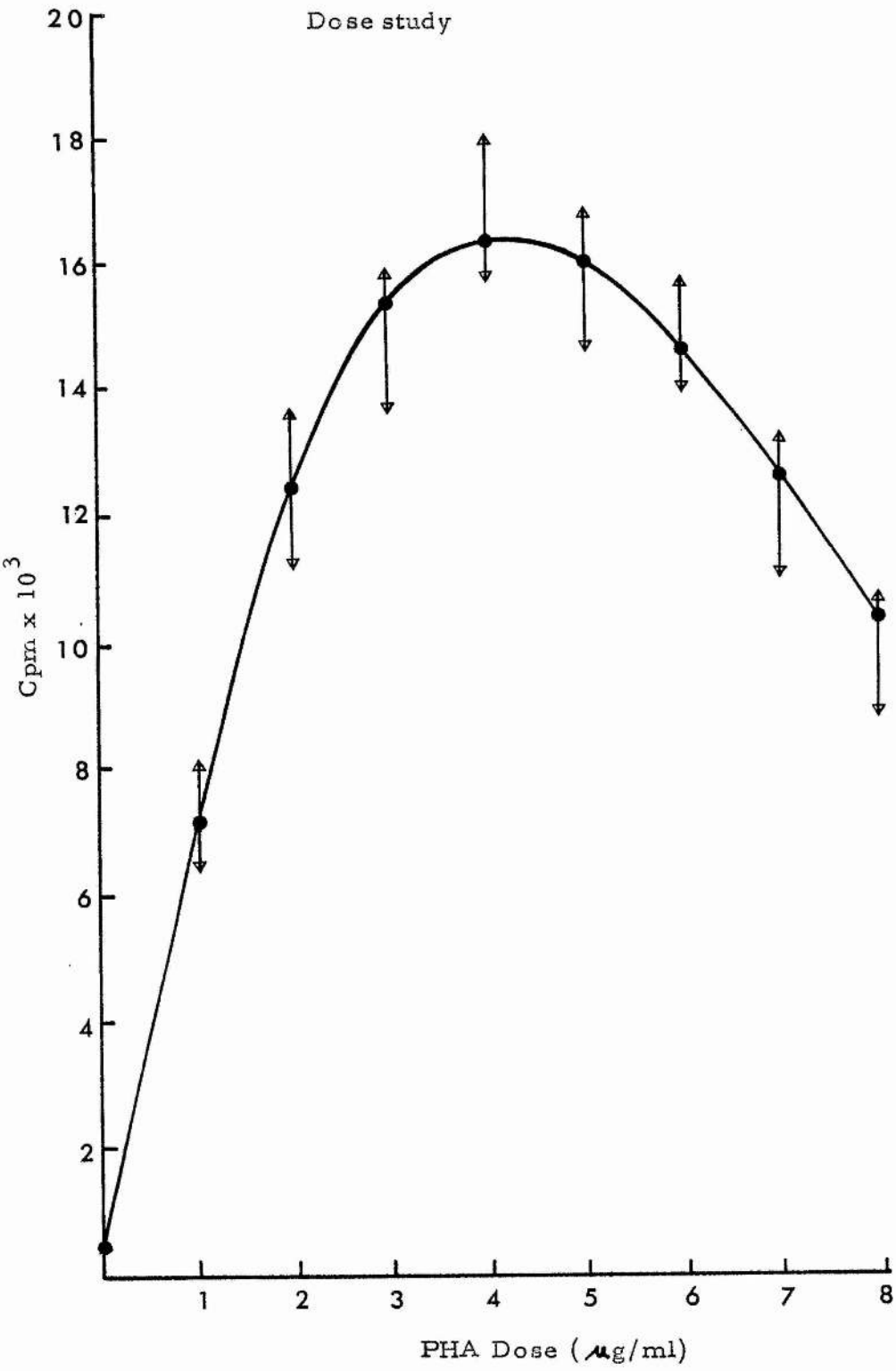


Table (1) Effect of conditioned medium on PHA response of thymocytes: Expt. (1)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	1528 ± 193	79	4.7 ± 1.3	< 0.001
Thymocytes/PEC CM + PHA	899 ± 108	44	2.8 ± 0.8	< 0.001
Thymocytes + PHA	555 ± 86	35	1.7 ± 0.5	< 0.001
Thymocytes only	324 ± 80	33		
Thymocytes + thymus CM	233 ± 33	13		
Thymocytes + PEC CM	241 ± 57	23		
Lymph node cells + PHA	14248 ± 283	115	50.3 ± 5.1	< 0.001
Lymph node cells only	351 ± 35	14		

Sample (N) = 6
Difference in stimulation induced by thymus and PEC CM ($p < 0.001$)

Table (2) Effect of conditioned medium on PHA responses of thymocytes : Expt. (2)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	1460 ± 179	73	5.4 ± 1.5	< 0.001
Thymocytes/PEC CM + PHA	1032 ± 173	71	3.8 ± 1.2	< 0.001
Thymocytes + PHA	672 ± 83	34	2.5 ± 0.7	< 0.001
Thymocytes only	268 ± 68	28		
Thymocytes + thymus CM	281 ± 56	28		
Thymocytes + PEC CM	205 ± 31	13		
Lymph node cells + PHA*	1176 ± 1029	420	31.1 ± 27.4	< 0.001
Lymph node cells only*	377 ± 43	18		

Sample (N) = 6 or *9
Difference in stimulation induced by thymus and PEC CM ($p < 0.001$)

Effect of conditioned medium on PHA response of thymocytes

Fig. 3

Expt. (1)

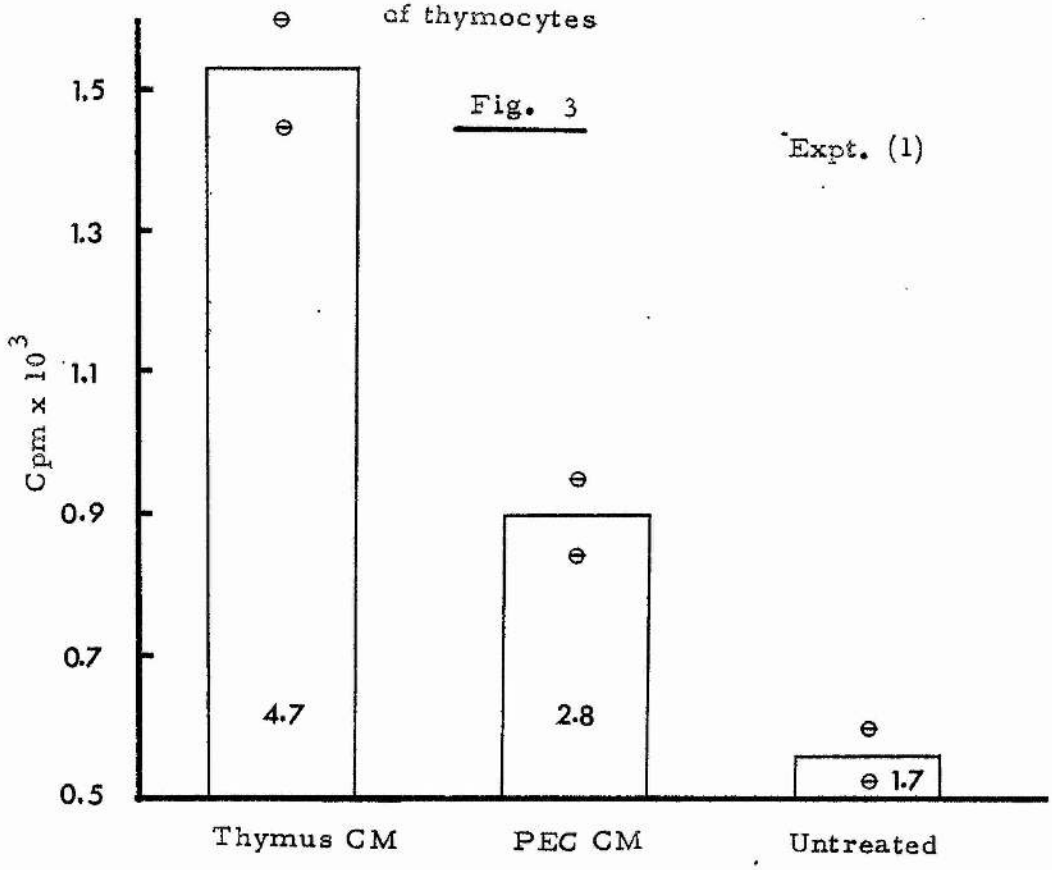
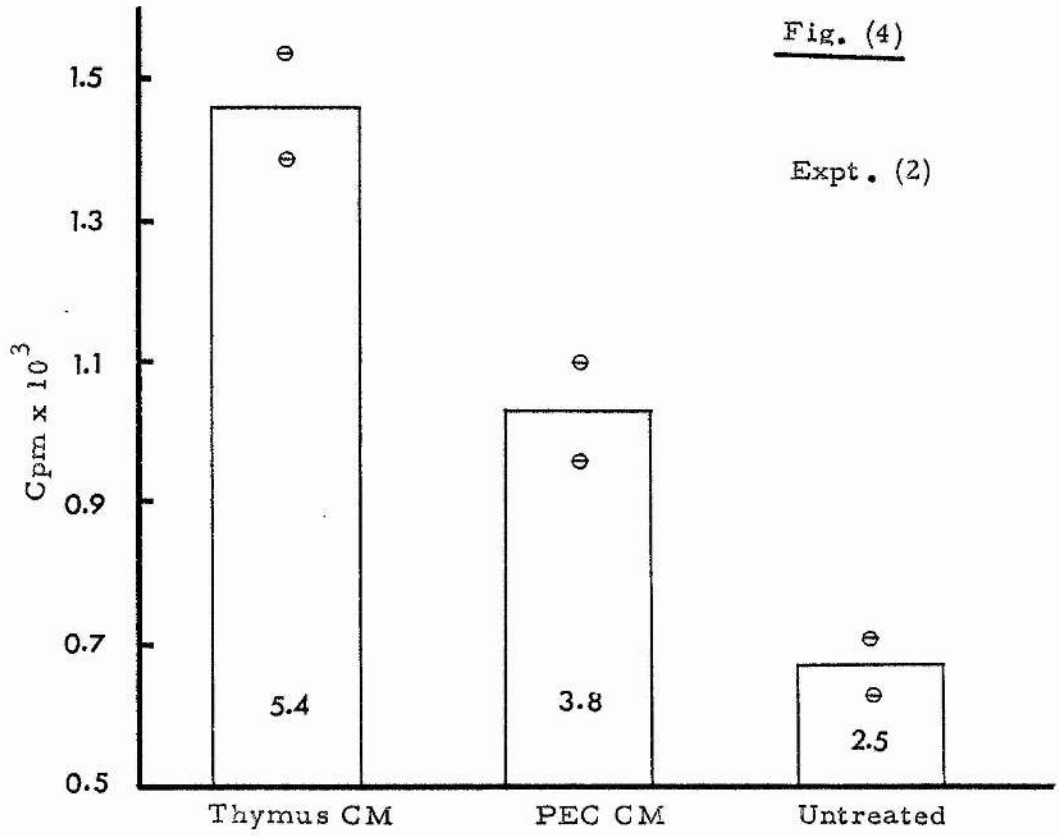


Fig. (4)

Expt. (2)



proliferation.

It is presumed that any active factors in the CM are derived by secretions of the monolayer cells. Therefore, the effect of incubation with CM collected after only 24 hours was tested. This would exert a very negligible effect at most if the hypothesis was true. Figs. (5) and (6) show the stimulation obtained with this CM. The scales on the axes are different for these two experiments from the earlier ones. The untreated cells responded to a lower than usual degree and consequently any differences appear exaggerated. The response obtained by the treated cells was of the same order as the control untreated cells in expts. (1) and (2). The CM in these experiments (1-4) was obtained from monolayers derived from young adult mice. Figs. (7) and (8) show data after incubation with CM collected after longer periods, from monolayers derived from foetal thymic tissue of 15 days gestation. The stimulation obtained is of the same order as in expts (1) and (2), although the absolute proliferative response is slightly lower.

In nearly all these cultures, the stimulation obtained with CM from PEC monolayers was greater than control untreated cultures, although less than that induced by thymus monolayers. In order to investigate the specificity of the inductive effect obtained by the CM, a dose study was carried out. Fig. (9) shows the data from expt (7) where the pre-incubated cells were cultured with PHA at doses between 1-4μg/ml. Maximal responses are obtained with 3 and 4μg/ml of PHA; stimulation indices of 5.4 and 5.1 were obtained with PHA at 4μg/ml with the CM from thymus and PEC monolayers respectively.

Table (3). Effect of conditioned medium on PHA response of thymocytes : Expt. (3)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	676 ± 174	71	3.4 ± 1.0	< 0.001
Thymocytes/PEC CM + PHA	489 ± 110	45	2.5 ± 0.6	< 0.01
Thymocytes + PHA	288 ± 86	35	1.5 ± 0.5	< 0.05
Thymocytes only	197 ± 25	10		
Thymocytes + thymus CM	127 ± 32	13		
Thymocytes + PEC CM	117 ± 22	9		
Lymph node cells + PHA*	12560 ± 967	395	34.7 ± 9.3	< 0.001
Lymph node cells only*	362 ± 93	38		

Sample (N) = 6 or *9

Difference in stimulation induced by thymus and PEC CM ($p < 0.02$)

Table (4). Effect of conditioned medium on PHA responses of thymocytes : Expt. (4)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	573 ± 116	47	2.7 ± 0.9	< 0.01
Thymocytes/PEC CM + PHA	327 ± 87	35	1.5 ± 0.5	> 0.1
Thymocytes + PHA	351 ± 60	24	1.7 ± 0.5	< 0.01
Thymocytes only	212 ± 52	21		
Thymocytes + thymus CM	193 ± 20	8		
Thymocytes + PEC CM	172 ± 73	30		
Lymph node cells + PHA*	11129 ± 768	313	30.7 ± 8.3	< 0.001
Lymph node cells only*	362 ± 93	38		

Sample (N) = 6 or *9

Difference in stimulation induced by thymus and PEC CM ($p < 0.001$)

Effect of conditioned medium on PHA response of thymocytes

Fig. (5) Expt. (3)

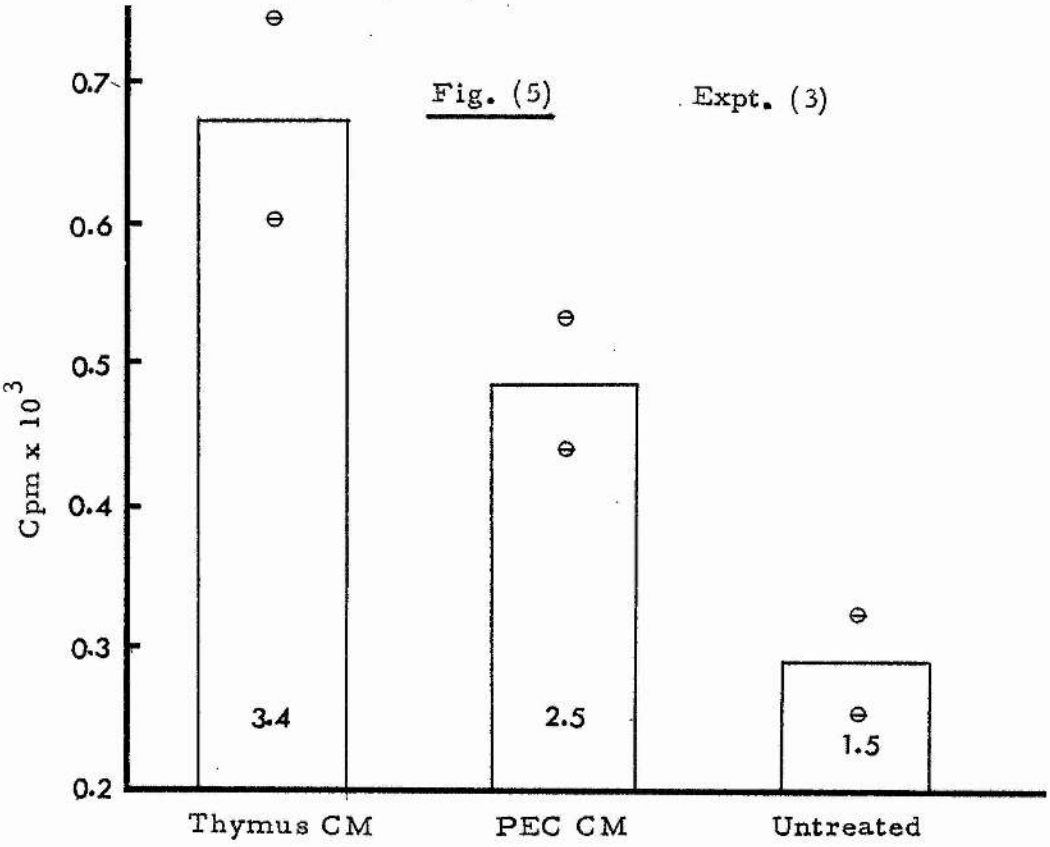


Fig. (6) Expt. (4)

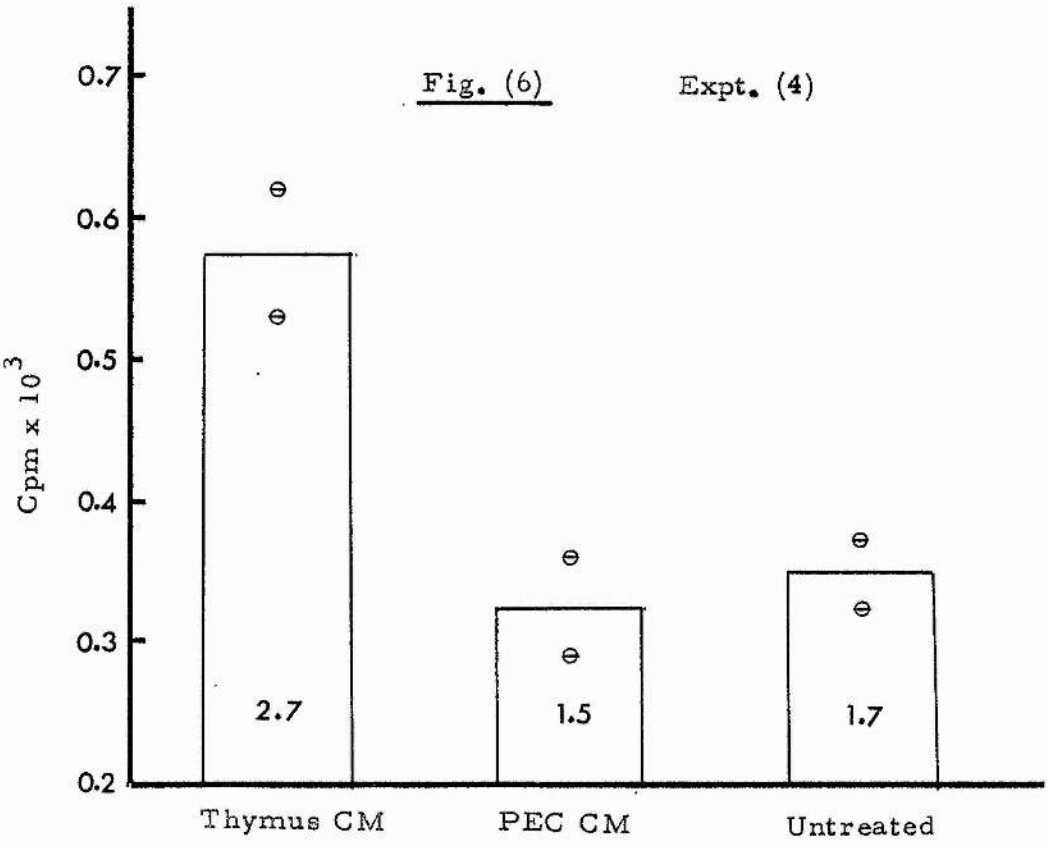


Table (5). Effect of conditioned medium on PHA response of thymocytes : Expt. (5)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	1173 ± 151	62	4.4 ± 1.5	< 0.001
Thymocytes/PEC CM + PHA	598 ± 183	74	2.3 ± 0.8	< 0.02
Thymocytes + PHA	329 ± 112	46	1.2 ± 0.6	> 0.1
Thymocytes only	269 ± 8.8	36		
Thymocytes + thymus CM	143 ± 7	3		
Thymocytes + PEC CM	73 ± 6	2		
Lymph node cells + PHA*	11299 ± 1433	585	39.5	< 0.001
Lymph node cells only*	286 ± 50	20		

Sample (N) = 6 or *9

Difference in stimulation induced by thymus and PEC CM ($p < 0.001$)

Table (6). Effect of conditioned medium on PHA response of thymocytes : Expt. (6)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM + PHA	896 ± 121	49	4.5 ± 1.1	< 0.001
Thymocytes/PEC CM + PHA	438 ± 112	46	2.2 ± 0.7	> 0.1
Thymocytes + PHA	357 ± 114	47	1.8 ± 0.6	0.01
Thymocytes only	197 ± 39	16		
Thymocytes + thymus CM	141 ± 15	6		
Thymocytes + PEC CM	93 ± 22	9		
Lymph node cells + PHA*	9880 ± 852	348	24.0 ± 5.9	< 0.001
Lymph node cells only*	407 ± 85	35		

Sample (N) = 6 or *9

Difference in stimulation induced by thymus and PEC CM ($p < 0.001$)

Effect of conditioned medium on PHA response of thymocytes

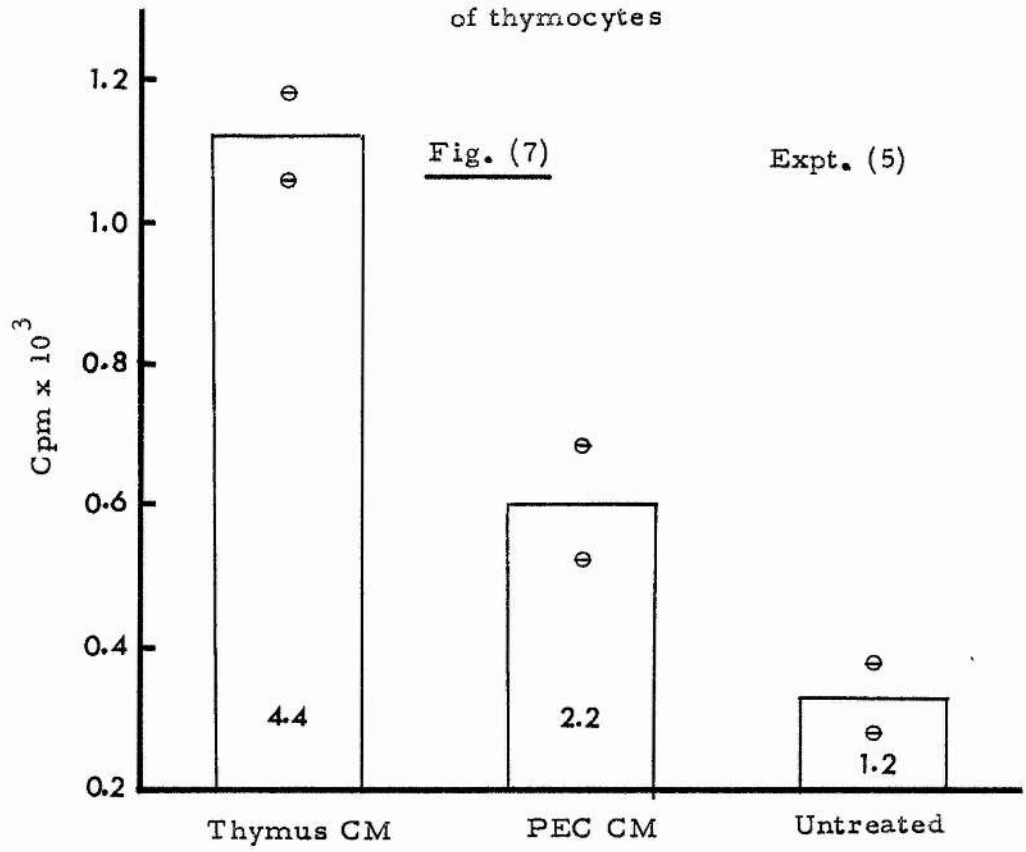


Fig. (7) Expt. (5)

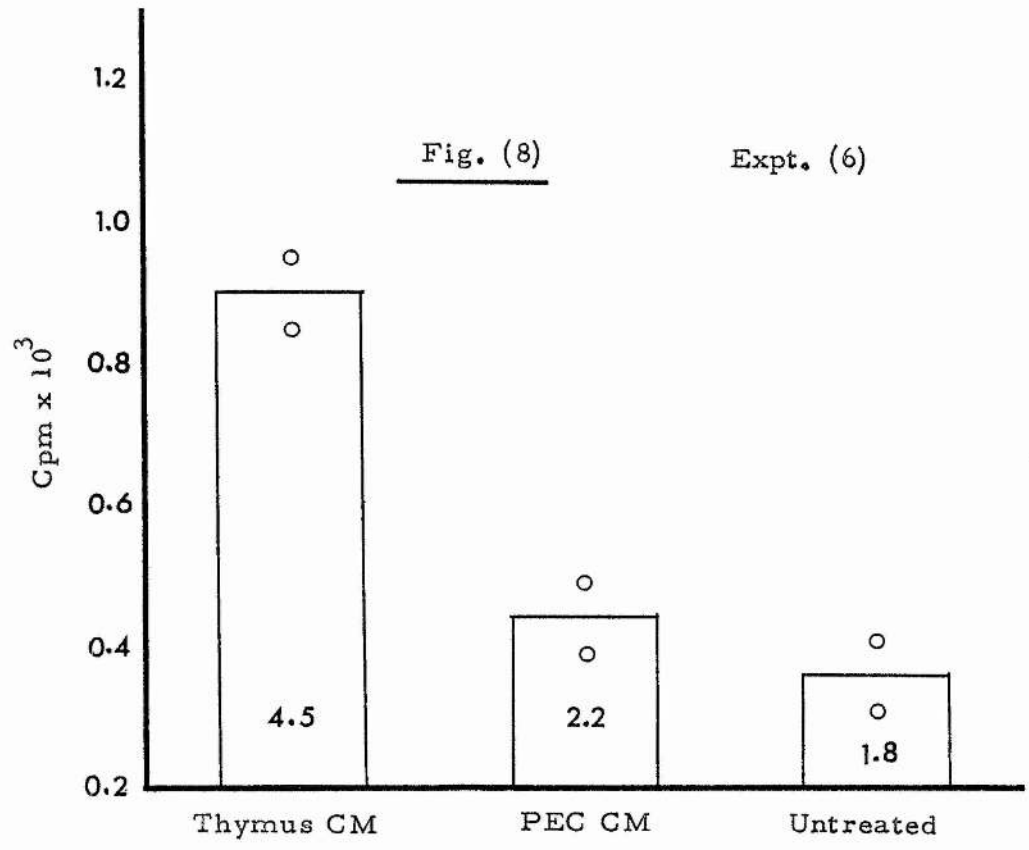


Fig. (8) Expt. (6)

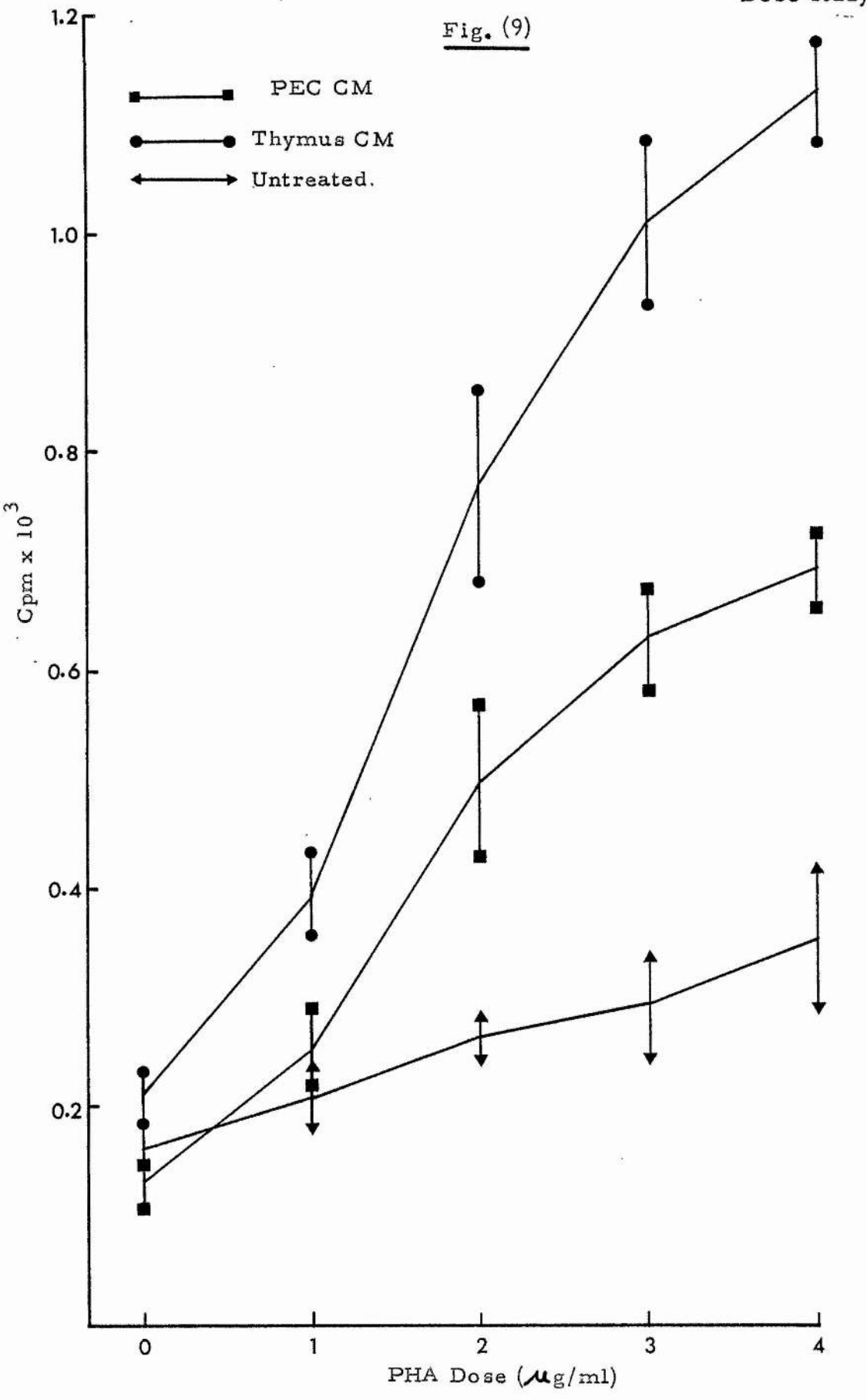
Table (7). Effect of conditioned medium on PHA response of thymocytes: Dose study Expt. (7)

Cells/Treatment	PHA Dose g/ml	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus CM	4	1132 ± 96	43	5.5 ± 1.5	<0.001
Thymocytes/thymus CM	3	1011 ± 161	72	4.8 ± 1.4	<0.001
Thymocytes/thymus CM	2	772 ± 186	83	3.7 ± 1.3	<0.001
Thymocytes/thymus CM	1	394 ± 73	33	1.9 ± 0.6	<0.01
Thymocytes/thymus CM	-	209 ± 53	23		
Thymocytes/PEC CM	4	693 ± 69	31	5.1 ± 1.8	<0.001
Thymocytes/PEC CM	3	632 ± 103	46	4.7 ± 1.8	<0.001
Thymocytes/PEC CM	2	501 ± 151	68	3.7 ± 1.7	<0.001
Thymocytes/PEC CM	1	253 ± 78	35	1.9 ± 0.9	<0.05
Thymocytes/PEC CM	-	135 ± 46	21		
Thymocytes only	4	353 ± 138	62	2.2 ± 1.4	<0.05
Thymocytes only	3	294 ± 88	39	1.8 ± 1.0	<0.05
Thymocytes only	2	267 ± 30	13	1.7 ± 0.9	<0.05
Thymocytes only	1	208 ± 61	27	1.3 ± 0.8	>0.1
Thymocytes only	-	160 ± 78	35		
Lymph node cells + PHA	4	11472 ± 1283	574	26.6 ± 4.1	<0.001
Lymph node cells only	-	431 ± 63	28		

Sample (N) = 5
Significant difference between thymus and PEC CM at all doses of PHA ($p<0.05$)

Effect of conditioned medium on PHA response of thymocytes: Dose study

Fig. (9)



(C) Effect of co-culture with monolayer cells

The PHA responsiveness of thymocytes after co-culture for 24 hours with thymic and PEC monolayers was investigated (Figs. 10 and 11). The open boxes represent co-cultured thymocytes stimulated with PHA and the closed boxes, unstimulated co-cultured thymocytes. The mitogenic stimulation obtained is generally slightly lower than that obtained by incubation with CM alone. In these experiments, the stimulation induced by co-culture with PEC monolayers approximated that obtained by thymus, as the stimulation indices were not significantly different.

(2) Expression of Thy 1 membrane antigen

Horse-radish peroxidase conjugated α-Thy 1 antisera was used to detect the Thy 1 antigen on different haemopoietic cell populations before and after co-culture with thymus monolayers. Cytocentrifuge preparations of the cells were fixed in acidified methanol to block endogenous peroxidase activity and then treated with the enzyme conjugated antisera.

Table (10) shows the data from this experiment. The numbers of Thy.1 bearing cells among the control lymph node cell population could not be increased after co-culture, as expected. Both normal and HN_2 treated bone marrow cells were found to show very negligible increases after co-culture. Spleen cells from nude mice were relatively more inducible and showed a 5.5% increase in Thy 1 bearing cells after co-culture with the monolayer cells. A hundred cells in each of ten slides were scored and the cells showing a uniform brown ring on their surfaces were considered as Thy 1 positive.

Table (8). Effect of co-culture with thymus and PEC monolayers on PHA response of thymocytes : Expt. (8)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus monolayer + PHA	797 ± 283	82	3.0 ± 0.7	< 0.001
Thymocytes/thymus monolayer	260 ± 87	25		
Thymocytes/PEC monolayer + PHA	626 ± 93	27	2.9 ± 0.7	< 0.001
Thymocytes/PEC monolayer	219 ± 44	13		
Thymocytes + PHA	332 ± 70	20	1.6 ± 0.4	< 0.001
Thymocytes only	207 ± 28	8		
Lymph node cells + PHA	8532 ± 1245	359	12.6 ± 0.4	< 0.001
Lymph node cells only	678 ± 201	58		

Sample (N) = 12

No significant difference between inductive effect by thymus and PEC monlayer cells (p 0.1)

Table (9). Effect of co-culture with thymus and PEC monolayers on PHA responses of thymocytes : Expt. (9)

Cells/Treatment ± PHA	Mean cpm ± SD	SE	SI ± SD	p
Thymocytes/thymus monolayer + PHA	1087 ± 292	84	3.9 ± 1.8	< 0.001
Thymocytes/thymus monolayer	279 ± 108	31		
Thymocytes/PEC monolayer + PHA	744 ± 206	59	3.3 ± 1.5	< 0.01
Thymocytes/PEC monolayer	228 ± 82	24		
Thymocytes + PHA	275 ± 71	20	1.4 ± 0.6	< 0.01
Thymocytes only	200 ± 22	6		
Lymph node cells + PHA	11248 ± 1633	471	26.7 ± 7.6	< 0.001
Lymph node cells only	420 ± 103	30		

Sample (N) = 12

No significant difference between inductive effect by thymus and PEC monolayer cells (p > 0.1)

Effect of co-culture on PHA response of thymocytes.

Fig. (10)

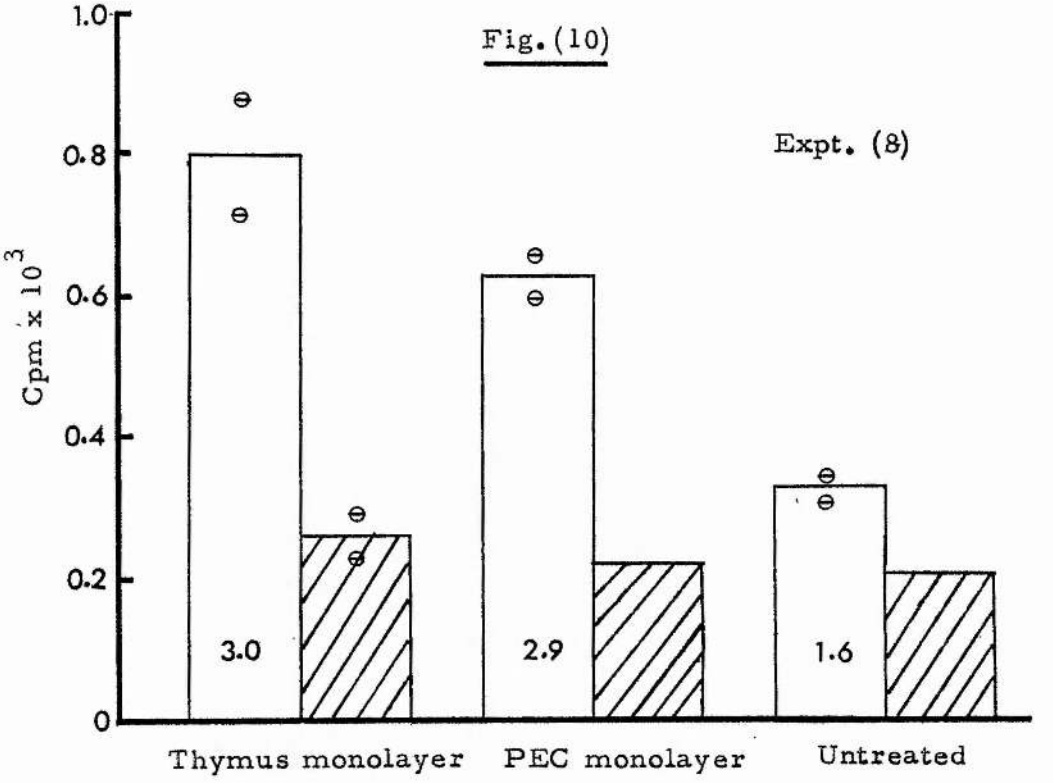
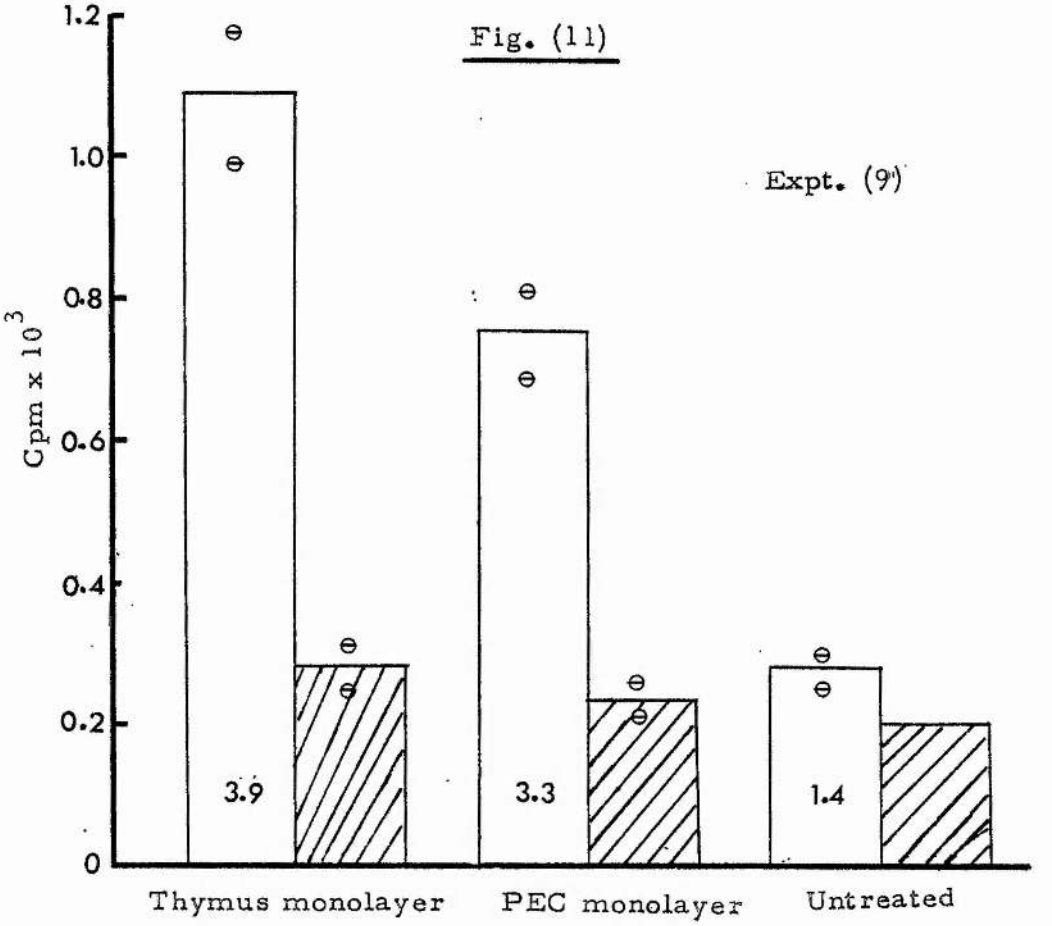


Fig. (11)



(3) Effect of co-culture and CM on sensitivity of thymocytes to testosterone

A calibration curve of the viability of thymocytes from young male mice to the cytotoxic effect of testosterone acetate after 15 minutes of incubation was plotted (Fig. 12). The concentration of testosterone used was between 0-0.2 mg/ml. The hormone, at a concentration of 0.025 mg/ml killed approx. 50% of the cells; this concentration was used in subsequent assays.

After 24 hours of co-culture with monolayers or incubation with their CM, the cells were incubated with the hormone and a viability test carried out. The data is shown in Table (12). As the hormone was initially dissolved in a small volume of alcohol, appropriate controls were included. It is seen that no marked effects on testosterone sensitivity are obtained with either of the treatments.

N.B.

Recovery of cells

Recovery of cells after incubation with CM or medium alone was similar in these experiments; over 60% of the cells were usually recovered. After co-culture, the recovery of cells varied between experiments, 50-80% of the initial inoculum was recovered. There were no marked differences between cell recovery from thymic and PEC monlayers. Cells incubated without a feeder layer survived poorly and less than 50% were recovered.

Table (10). Effect of co-culture with thymus monolayers on expression of Thy.1 antigen

Cells	Cells	% Thy.1 positive cells ± SD Before co-culture	After co-culture
Lymph node		72.9 ± 10.7	69.8 ± 9.6
Normal bone marrow		5.6 ± 3.2	7.0 ± 3.4
HN_2 treated bone marrow		3.5 ± 2.4	4.1 ± 2.1
Bone marrow lymphocytes		3.8 ± 2.1	4.2 ± 2.3
Nude mouse spleen		2.8 ± 2.3	8.3 ± 2.8

Sample (N) = 10

Increases obtained with nude mouse spleen cells after co-culture ($p < 0.001$). No differences in all other cell populations ($p > 0.1$).

Table (11). Viability of thymocytes after incubation with testosterone acetate: Dose study

Sample (N) = 10

Conc. of hormone mg/ml	% Viability ± SD
0	91.0 ± 4.8
0.025	44.3 ± 6.8
0.05	26.3 ± 5.6
0.1	7.4 ± 4.2
0.2	0

Table (12). Effect of co-culture with thymus monolayers and incubation with conditioned medium on sensitivity of thymocytes to testosterone

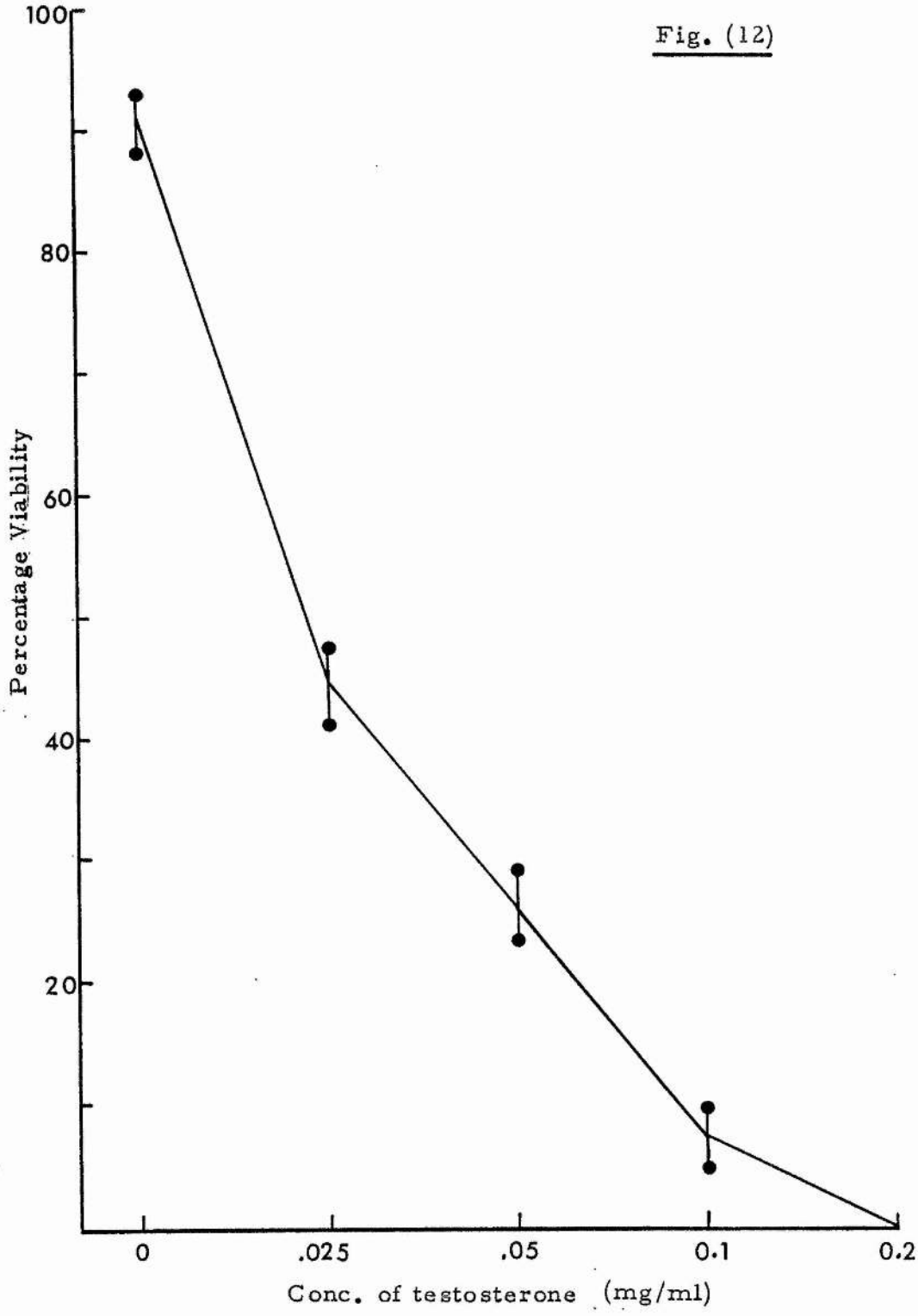
Treatment	% Viability after incubation in: Testosterone	Alcohol (control)
Co-culture with thymus monolayer cells	43.2 ± 10.0	85.5 ± 8.6
Incubation with thymus CM	46.9 ± 8.3	89.8 ± 3.2
Incubation with medium only	53.1 ± 4.3	93.1 ± 4.1

Sample (N) = 10

No difference in sensitivity to testosterone after either treatment ($p > 0.1$)

Viability of thymocytes after incubation with testosterone

Fig. (12)



DISCUSSION

The monolayers of cultured thymus cells, though heterogenous, are morphologically similar to those described by other investigators. The monolayers described by Mosier and Pierce (1972) were similarly derived by teasing apart intact thymic tissue. Waksal et al (1975) obtained their monolayers by prior overnight enzymic digestion with collagenase followed by trypsin. Early in this study, thymic tissue was similarly treated with these enzymes for shorter periods; the resulting suspension was a gelatinous mixture which did not prove convenient for culturing, although this could be rendered more suitable by the use of DNase. These investigators obtained confluent monolayers by 10 days, whereas confluence was only reached in nearly a month in this study. At the ultrastructural level, their cells were similar to that cultured in this and other laboratories ie. large, irregular cells with numerous cytoplasmic granules. The presence of free ribosomes possibly associated with endoplasmic reticulum containing cisternae were seen. Sato et al (1976) also obtained their monolayers from enzyme digested tissue and furthermore only secondary cultures were used in their induction assays. In this study, it was never possible to maintain secondary cultures for more than a few days. Loor (1979) obtained enzymically dispersed cells which were then fractionated further on a discontinuous bovine serum albumin (BSA) gradient. He obtained an enriched reticuloepithelial cell population which was subsequently cultured. Many of his cells had a granular cytoplasm and were binucleated. Such binucleated cells with prominent dense nucleoli were regularly seen in this study. At the ultrastructural level, his cells had many

other features in common, such as granular cytoplasm, abundant ribosomes and mitochondria and a well developed golgi apparatus. However, interestingly, desmosomes were not detected in his cultured cells. He detected the presence of fat globules by u.v. illumination in the light microscope which probably correlates with the intensely osmiophilic structures regularly seen in EM preparations of cultured cells. In Loor's culture technique, a further modification was introduced, he added triiodothyronine to enhance epithelial cell growth.

In other investigations, thymic monolayer cells cultured from non-murine sources have been claimed to be epithelial. Willis-Carr et al (1978) found that less than 1% of their monkey and human cultured cells were macrophages, as assayed by latex particle ingestion. The thymic monolayers derived from rats appear to have more typical epithelial cell characteristics. Kruisbeek et al (1977) detected desmosomes and tonofilaments in their cultured cells, they also claim to find morphological evidnce of active protein synthesis and export. The CM from such rat monolayer cultures, at a fairly high dilution of $^{1}/_{20}$ has been shown to increase the mitogen responsiveness of cortisone sensitive rat thymocytes. Incubation with such CM caused enhanced in vitro antibody production to SRBC by nude mouse spleen cells, raised endogenous cAMP levels and reactivity of thymocytes in the MLC. The CM from human thymus monolayers have been similarly shown to enhance mitogen responsiveness of human and mouse thymocytes by Oosterom et al (1979); but their thymocytes were obtained from cortisone treated animals which are therefore enriched for a

relatively mature thymocyte population. These authors, like others have characterised their cells as being epithelial on the basis of finding desmosomes and tonofilaments, though few have presented morphological evidence in support.

In the co-culture experiments, a proportion of thymocytes were at times difficult to dislodge from the feeder cells. Vigorous squirting with pasteur pippettes was sometimes necessary. The recovery of cells varied enormously and sometimes viability of recovered cells was poor. It is possible that poor recovery is a consequence of thymocytes being phagocytosed by the monolayer cells during culture. During the FcR assay, if the incubation period with sensitised ox erythrocytes was prolonged, they were found to be engulfed by the monolayer cells. As the thymocytes were co-cultured for 24 hours, some may well have suffered a similar fate. If the thymocytes are sensitised in vitro as suggested by Wekerle et al (1973), this could result in uptake of the isotope as a consequence of blast formation due to factors other than the mitogenicity of PHA. When thymocytes were pre-incubated with the CM from thymus cultures, the cell recovery was more consistent. In addition, several batches of CM could be tested and used again if proved active, whereas a monolayer had a limited lifetime in culture. However, in both these systems, the possible effects of xenogeneic thymus derived factors in the calf serum supplements have to be considered. Thymosin is usually prepared from calf thymus; on the other hand, a low m.wt. inhibitor of circulating thymic factor has been found in human serum (Bach and Dardenne, 1973). Any similar factors in calf serum could adversely affect potential inductive effects.

In both these systems, the increases in PHA reactivity of the treated thymocytes are limited and less than that obtained by some investigators. The increases seen varied with different experiments and a stimulation index of 5.5 was the highest obtained when compared to unstimulated cells, or 2-3 if compared to untreated stimulated cells. However, ^{125}IUdR was used as the labelled DNA precursor in this study, whereas ^{3}HTdR was generally employed in others.

It is perhaps surprising that a monolayer composed predominantly of macrophages is able to cause any maturation at all in the target cell population. However, the recent accumulation of data on the role of macrophages and their secreted factors in modulating immune reactivity puts these findings in a different context (to be discussed later). The findings of Beller and Unanue (1977 and 1978) and of Van den Tweel and Walker (1977) are relevant to the present observations. These investigators found that immature thymocytes could be induced to differentiate by co-culture with thymic macrophages or their culture fluids. Increased expression of H.2 antigens, decreased sensitivity to α-TL + C' and acquired responsiveness to mitogens were detected in these cells. In this study, co-culture with monolayers derived from PEC or their CM had a lesser but noticeable effect on the PHA responsiveness of thymocytes, compared to thymus derived monolayers. It is not known whether dislodged monolayer cells are obtained as well during recovery of thymocytes after co-culture; if so, they may well contribute in a manner suggested by these authors.

It is difficult to compare the limited inductive effects obtained in this study with the results of other investigators, most of whom have used a xenogeneic system. Mosier and Pierce (1972) investigated the effects of co-culture of mouse thymocytes upon syngeneic monolayers. Their inductive effects were much greater than that seen in these experiments; the reactivity of their cultured thymocytes equated with that of peripheral lymphocytes in the MLR, responsiveness to phytomitogens and helper function. Their period of culture was much longer and they identify their putative epithelial cells as being PAS negative, whereas a few glycogen containing large cells were found in this study. It is interesting that these investigators also obtained some degree of induction with adherent cells from the spleen, presumably macrophages.

The morphological and functional characterisation of the monolayer cells cultured in this study confirm the earlier findings of Jordan et al (1979a) and Harper and Sharp (in press), of the predominant macrophage composition of cultured thymic stromal cells. Attempts to determine whether these cells possessed surface FcR gave inconsistent results. Isolated erythrocytes were found adhered to their surfaces unlike the tight clusters seen in more typical rosettes. However, if there are a limited number of FcR sites per cell, these would become scattered as a consequence of the cell spreading out on the substrate. Thus, any attached erythrocytes would appear diffusely scattered because of the cell spreading out on the substrate, as seen in this study, assuming that bound FcR do not cap. The majority of PEC in early cultures formed rosettes; whereas in older cultures where they

were more spread out, the attached erythrocytes were diffuse as seen with thymus monolayers.

The levels of mitogen responsiveness acquired in these co-cultured experiments are of the same order obtained by Harper and Sharp, though these investigators have used bone marrow and spleen cells from athymic and thymus-deprived mice as their target cell populations; in addition, they used ^{3}HTdR incorporation as a measure of proliferation. Jordan et al(1979b) have grafted the cultured cells in vivo and obtained repopulation with host stem cells; this suggests that although macrophages are the predominant cells in culture, the epithelial component is still prevalent. In this study, the very low numbers of cells recovered by trypsinisation from the culture flasks severely limited any such attempt to recreate the thymic environment in vivo.

The co-culture of haemopoietic cells with thymic monolayers did not induce the expression of the Thy.1 antigen on these cells, with the possible exception of spleen cells from nude mice. An approx. three-fold increase was seen with these cells. Papiernik et al (1975), using human thymic epithelium found that spleen cells from thymus deprived mice were rendered sensitive to α-Thy 1 + C' after co-culture as detected by the E-rosette assay. The Thy 1 antigen detected on 5.6% of normal bone marrow cells most probably represents the recirculatory mature T cells in that population. However, HN_2 treated marrow had a slightly lower number of Thy 1 bearing cells, suggesting that it probably contains some post-thymic T cells, although in reduced numbers. Bone marrow lymphocytes from density gradients had a similar number of Thy 1 positive cells. This method of enriching marrow

lymphocytes has been used by Press and Rosse (1977a) who find their cells to be Thy 1 negative (Chapter 3). It is of course possible that haemopoietic pre-thymic cells which are known to have H.2 antigens also have the Thy 1 antigen; spleen cells from athymic mice are thought to have low but detectable levels of Thy 1. Van den Engh et al (1978) have detected H.2 as well as an antigen associated with brain tissue on multipotential stem cells. A heteroantisera such as the one used in this study could very well cross-react with determinants on antigens other than Thy 1.

It is accepted that cortisone resistance of medullary thymocytes is a consequence of a maturational event in cortisone sensitive cortical cells in vivo. Sensitivity of thymocytes to the sex-steroid testosterone in vitro was not found to be affected after prior exposure to thymic monolayers or their supernatants. Astaldi et al (1977) have found that a factor from human serum was able to enrich the cortisone resistant population of mouse thymocytes upon incubation.

This study has confirmed the recent findings of other investigators that culture of stromal cells from mouse thymus as monolayers, does not enrich for epithelial cells as has been believed previously. Any degree of lymphoid differentiation induced by these cells through their interaction or secreted soluble factors on haemopoietic cells is minimal.

CHAPTER 2

Organ culture of embryonic thymus

INTRODUCTION

The early literature describes the use of tissue culture techniques for investigating the histogenesis of the thymus (Emmart, 1936); lack of success in obtaining lymphoid development being attributed to poor culture conditions. Tissue culture of organ explants on inert strata such as lens paper (Chen, 1954) or modified by support on metal grids (Trowell, 1954) have provided a useful means of studying cellular growth and function, without disruption of the integrity of the tissue.

Auerbach (1960 and 1961) improved the techniques and obtained lymphoid development in vitro, and Ball and Auerbach (1960) utilised a modified filter well method to culture embryonic thymus. Owen et al (1974) further modified the system to culture foetal liver explants on membrane filters floating on culture medium at an interface of humidified 5% CO_2 in air. Surface immunoglobulin bearing lymphoid cells were found to differentiate in vitro, suggesting that the mammalian bursa-equivalent might be the liver during embryonic life. Robinson and Owen (1976 and 1977) have utilised this system to study the ontogeny of immunocompetence in foetal thymus, and furthermore to generate tolerance in vitro; the system has the advantage of investigating thymocyte maturation in an essentially closed system. Tufveson et al (1976) and Juhlin et al (1978) have similarly studied the ontogeny of mitogen responsiveness and acquisition of MLC reactivity in cultured embryonic mouse thymus. These investigators have utilised the system to promote lymphoid development in vitro.

In this study, an organ culture system was employed to selectively deplete endogenous lymphoid precursors in foetal thymic explants in order to obtain an enriched population of epithelial or reticulum cells. It was intended to reintroduce a population of lymphoid precursor cells into an epithelial, alymphoid thymus and subsequently culture the reconstituted organ to promote lymphoid development. It was envisaged that such an experimental protocol would enable the thymus seeding capacity of purified populations of haemopoietic cells to be evaluated. This would in turn provide some insight into any possible diversity within the stem cell populations committed to the T lymphocyte lineage of differentiation. A possible cellular candidate for the proposed reconstitution experiments would be the bone marrow cells from mice previously treated with nitrogen mustard (HN_2). Another cell population that could be investigated for its thymus repopulating potential would be that fraction of pre-thymic bone marrow lymphocytes isolated by sucrose density gradient centrifugation (Press and Rosse, 1977a and Chapter 3). Any such cellular reconstitution in vitro across the hisocompatability barrier might possibly elucidate when if at all, tolerance could be induced during T cell maturation in an allogeneic thymic environment. A thymus devoid of lymphoid cells would also provide a suitable source of enriched epithelial cells for culture as monolayers (Chapter 1). The rationale for using foetal thymic tissue was primarily because of its relative sparsity of lymphoid cells compared to the adult organ, and its small size which renders it suitable for organ culture in its entirety. Furthermore, foetal tissue still being in the growth phase may prove more adaptable to conditions in vitro.

PROCEDURE

1. Organ culture techniques

Details of the organ culture techniques are described in detail in the Materials and Methods section; only those experimental protocols that were employed to favour depletion or amplification of the lymphoid cell population will be described in this subsection.

(A) The culture conditions described by Robinson and Owen (1976) with few modifications were applied to favour lymphoid development in the foetal thymus. The explants were cultured in RPMI medium containing 10% FCS in a humidified atmosphere of 5% CO_2/95% Air.

(B) In order to deplete the explants of their endogenous lymphoid cells, a variety of culture conditions were tested, which included the use of serum supplement and the gaseous composition of the atmosphere. The optimal culture conditions for inhibiting lymphoid development, yet permitting epithelial growth was found to be initial culture of the explants in Waymouth's medium without serum, under a humidified atmosphere of 5% CO_2/95% O_2. The medium was replaced 48-72 hours later with either Waymouth's or RPMI medium, but both containing 10% FCS. The gaseous composition was maintained as before. The timing was not found to be critical, as cultures were not adversely affected if the conditions were changed up to 72 hours after start of the culture. Approximately 4 days under these conditions was sufficient to deplete individual lobes of 15 day foetal thymus. Larger explants were maintained for at least 6 days under similar conditions to deplete their lymphoid cells.

2. Reconstitution of lymphoid depleted thymus

(A) Lymphoid depleted explants were injected with suspensions of haemopoietic cells and subsequently cultured in conditions to promote lymphoid development, as described previously. Injections were carried out with glass capillary tubes or microsyringes containing either bone marrow cells from HN_2 treated mice or normal bone marrow isolated on sucrose density gradients (Chapter 3). In addition, thymocytes from untreated 15 day foetal thymuses mechanically teased apart were used. These cells were injected into composite explants comprised of 4-6 individual foetal thymus lobes fused together. These were obtained by co-culturing individual explants closely apposed to each other for 6 days under conditions inhibitory for lymphoid growth.

(B) Haemopoietic cells were reintroduced into lymphoid depleted composite explants by fusion with an untreated 14 day foetal thymic rudiment. The process was similar to that described previously, except under conditions to promote lymphoid development. After fusion, migration of lymphoblasts from the younger to the temporally older explant occurs, thus repopulating the latter.

(C) In a further attempt to reconstitute the individual cellular components of the thymus in vitro, trypsinised foetal thymic tissue was re-aggregated in agar tubes and subsequently cultured under conditions to promote lymphoid growth.

RESULTS

In the subsequent text, the term "explants" refer to single lobes of embryonic thymus at 15 days of gestation, unless otherwise stated. Plates (20) and (21) are photographs taken from published drawings of histological sections of human foetal and newborn calf thymus respectively by Hammar (1905).

Plates (22)-(34) are photomicrographs of histological sections of untreated foetal thymus from 14-17 days of gestation, and the corresponding cytocentrifuge preparations of cells teased from the organ. These are included to illustrate the histogenesis of the organ *in vivo* in late embryonic life when lymphopoiesis is most active. They are also included to compare the morphology of thymus explants after varying culture conditions and experimental protocols, which comprise the rest of the data of this chapter.

Although it was possible to detect the foetal thymus under a dissecting microscope at 13 days of gestation, its extirpation and subsequent experimental manipulation posed enormous technical difficulties. These problems could be overcome with 14 day organs; however the embryonic thymus at 15 days appeared as a paired structure and could be confidently removed without other contaminating tissue and manipulated experimentally with ease. At 14 days gestation, the organ is composed of a few darkly stained basophilic cells interspersed apparently randomly among the pale-staining epithelial cells of the rudiment (Plate 23). There does not appear to be a well-marked cortical/medullary organisation in the embryonic organ as in the adult. Cells teased apart mechanically from the organ are predominantly lymphoblasts and not epithelial cells, as seen in the cytocentrifuge preparations

Plate (20) Drawing of a section from a 70 mm human foetal thymus.

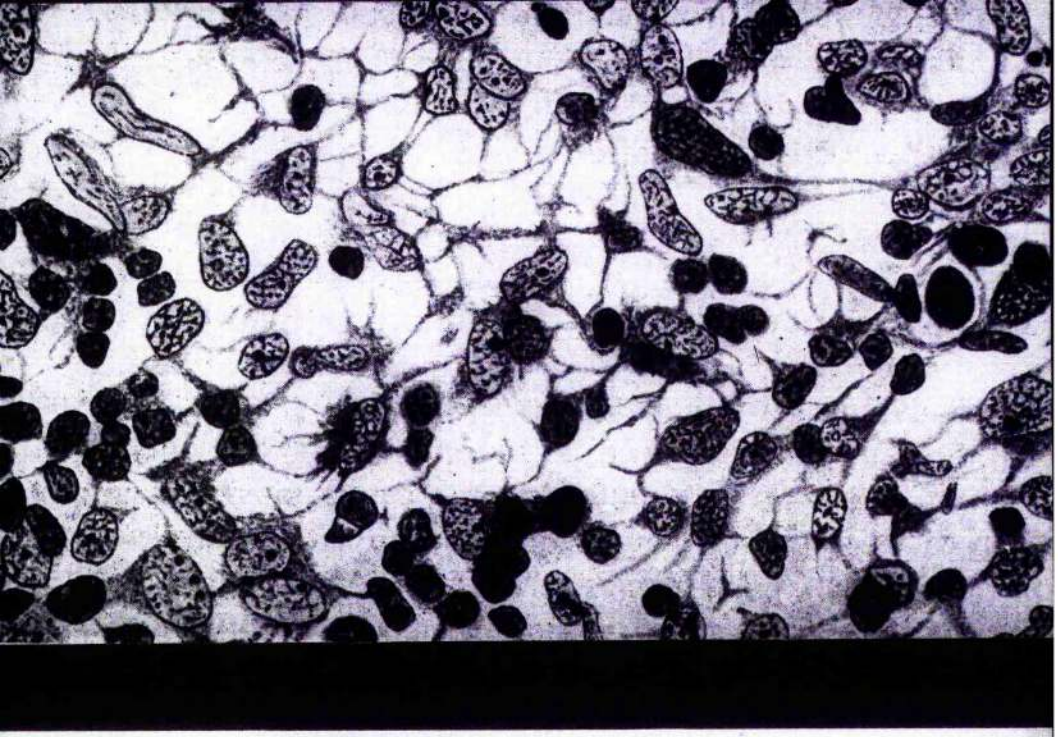
(From Hammar, 1905)

Plate (21) Hassall's corpuscle in newborn calf thymus.

(As above)

Plate (22) Thymus from 14 day mouse embryo.

Haematoxylin and Eosin (H and E) x 120



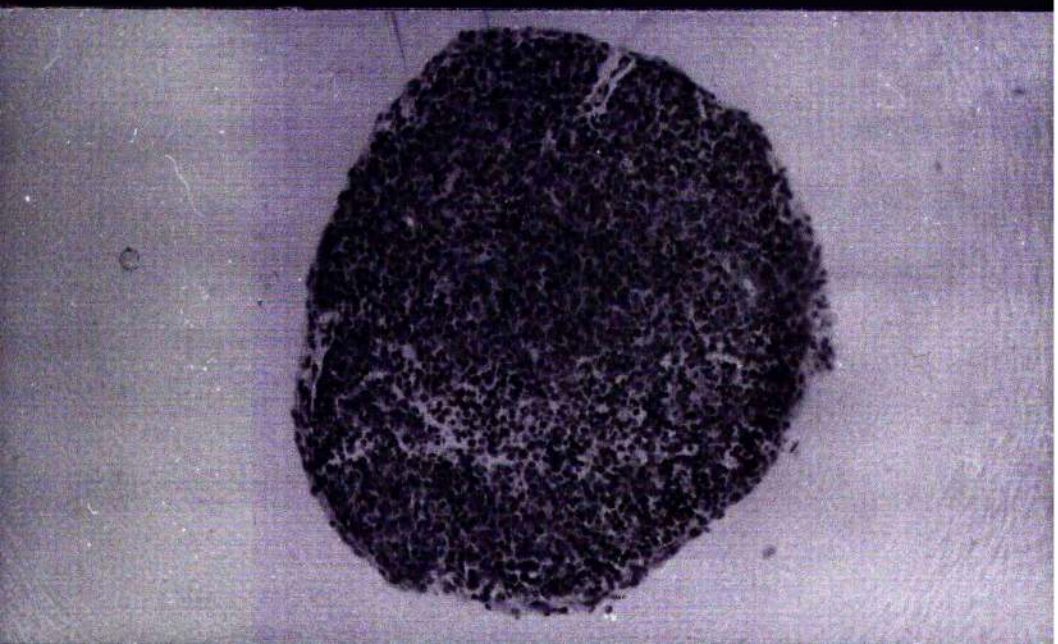


Plate (23) Thymus from 14 day mouse embryo. Few darkly staining basophilic lymphoid cells are seen amongst the paler epithelial reticulum of the organ.

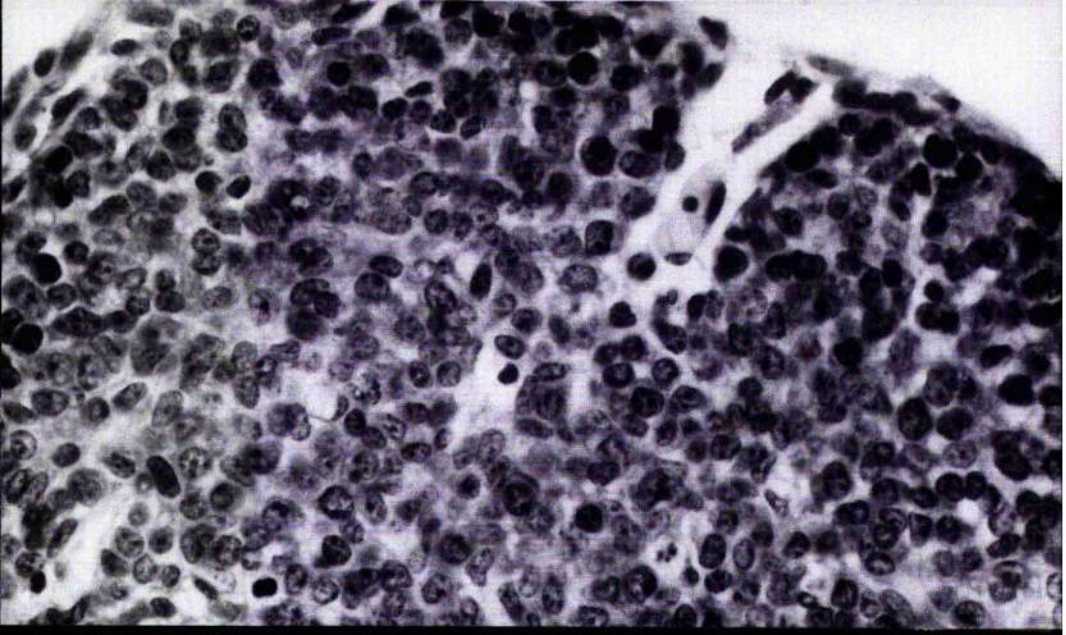
H and E x 480

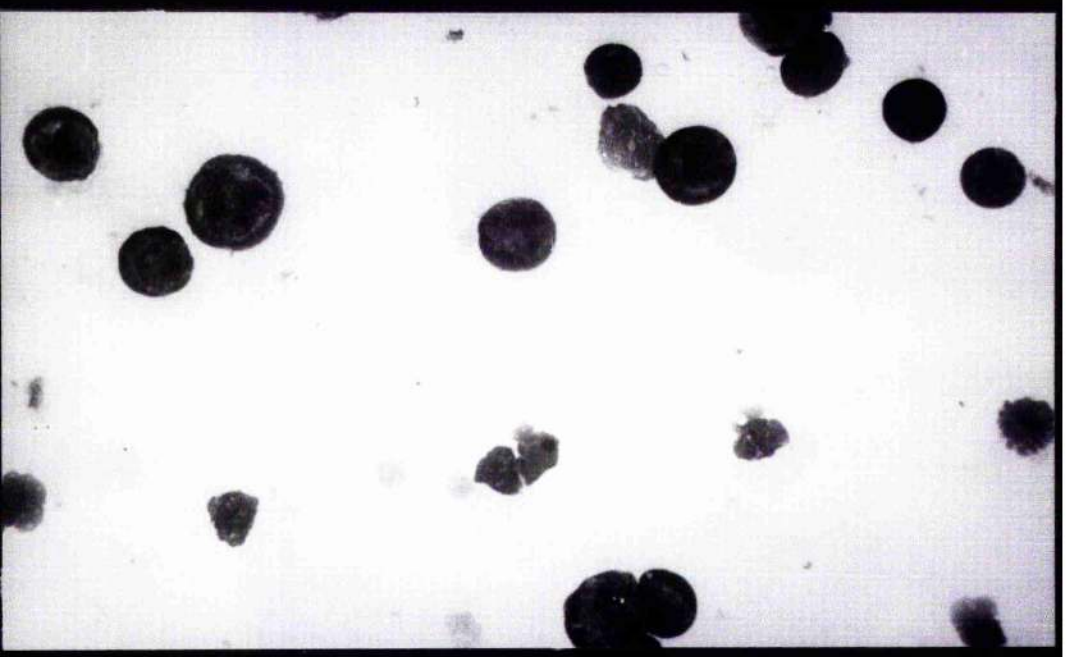
Plate (24) Cytospin preparation of lymphoid cells from above which are predominantly lymphoblasts.

J and G x 1200

Plate (25) Thymus from 15 day mouse embryo.

H and E x 120





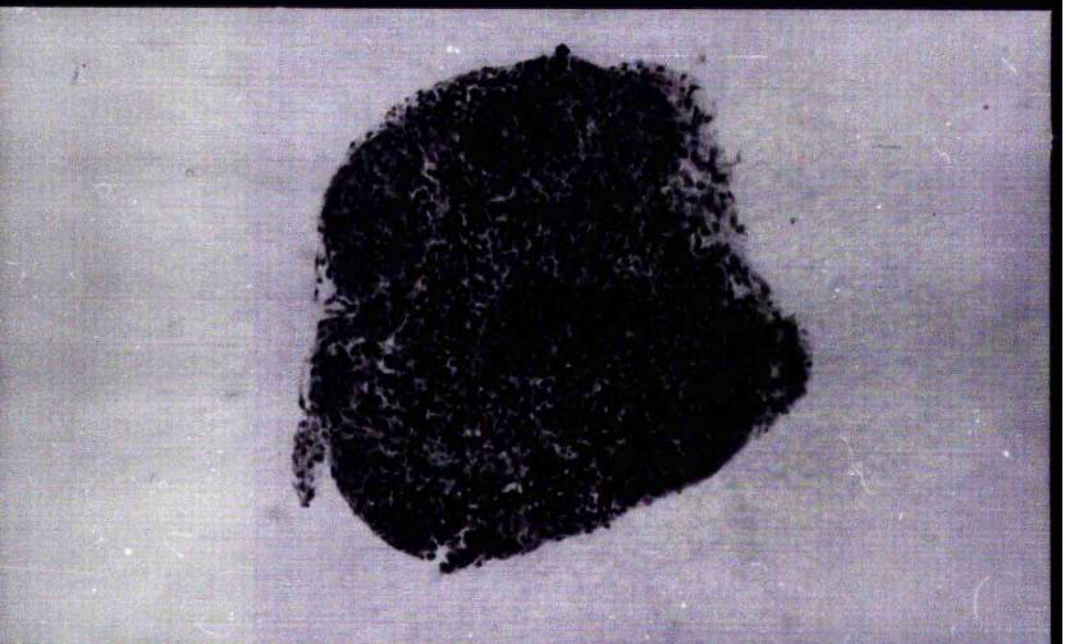


Plate (26) Thymus from 15 day mouse embryo.

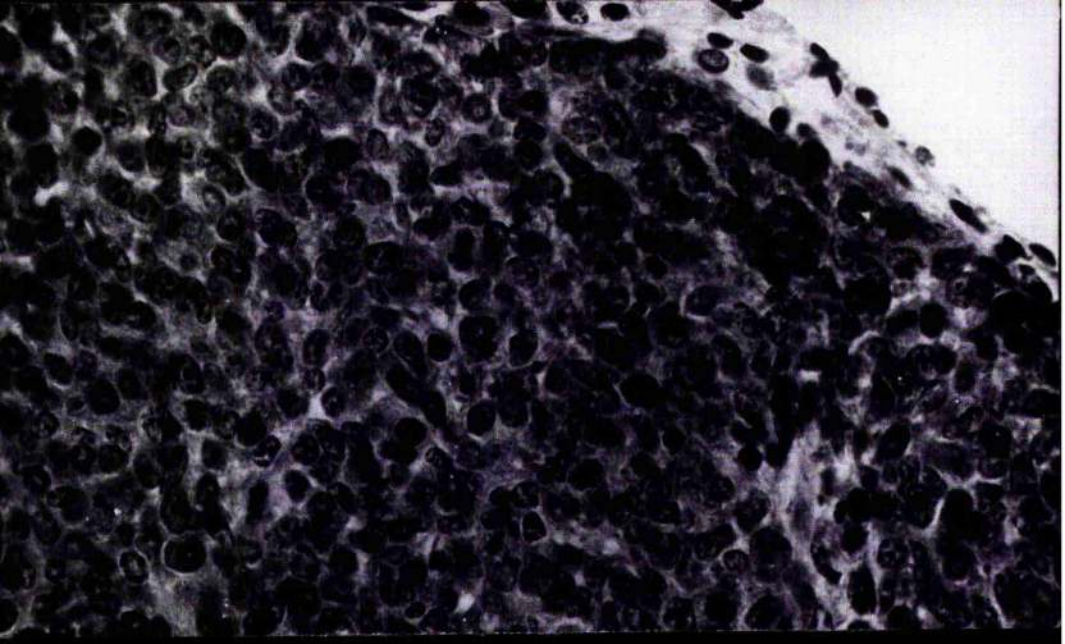
H and E x 4800

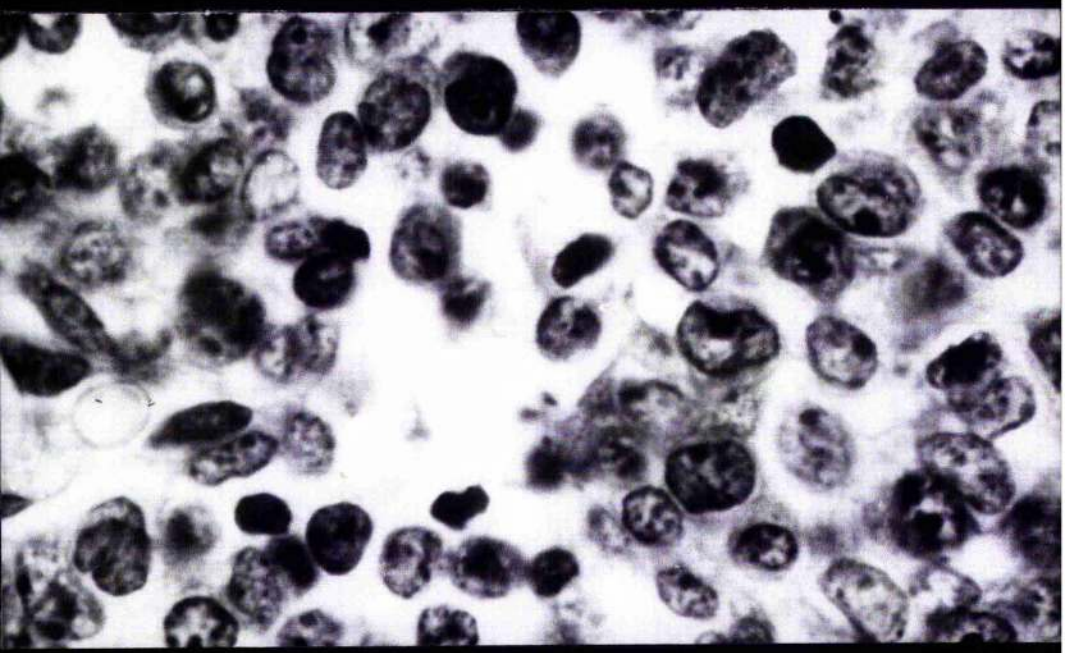
Plate (27) Higher magnification of above.

H and E x 1200

Plate (28) Cytospin preparation of lymphoid cells from above.

J and G x 1200





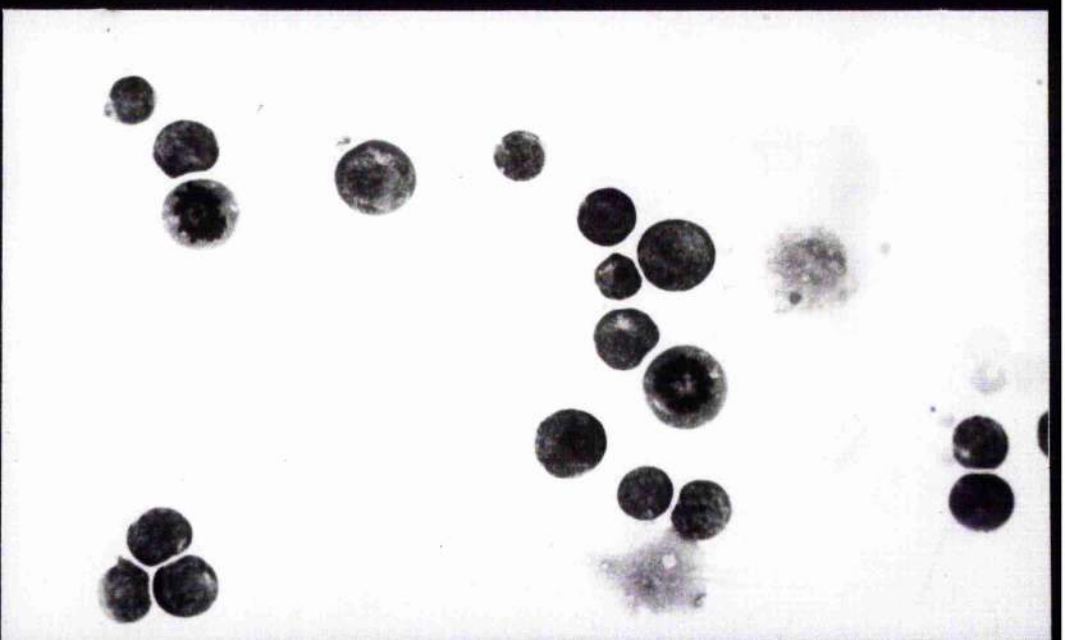


Plate (29) Thymus from 15 day mouse embryo. The organ is evidently more lymphoid than at earlier stages of gestation.

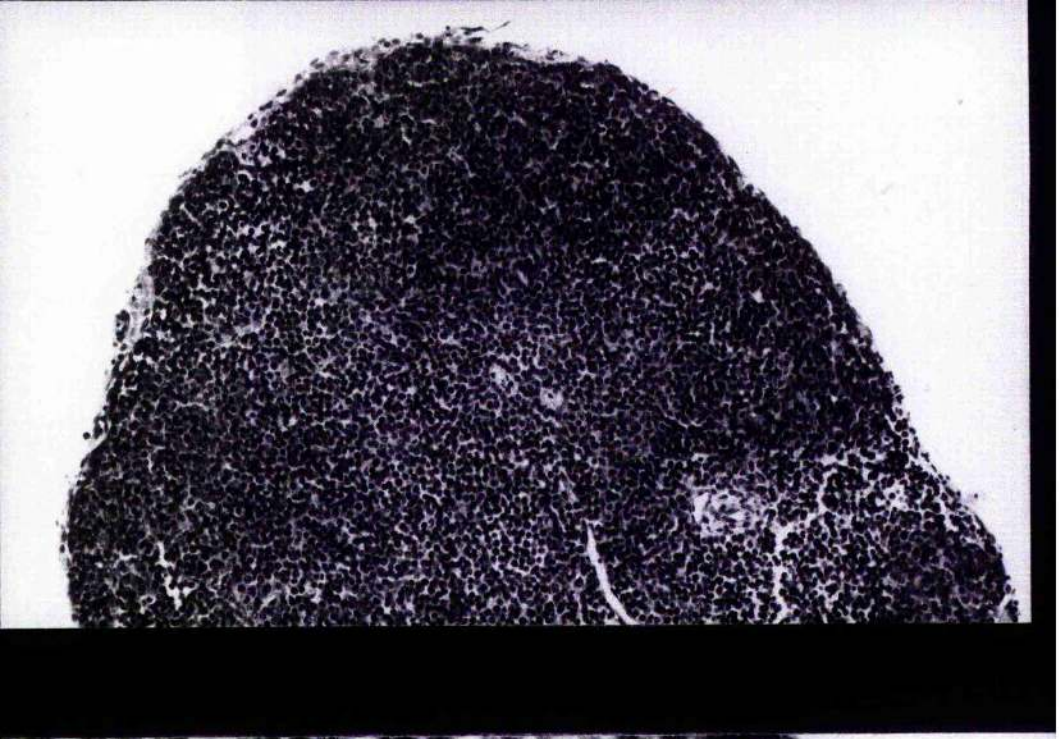
H and E x 120

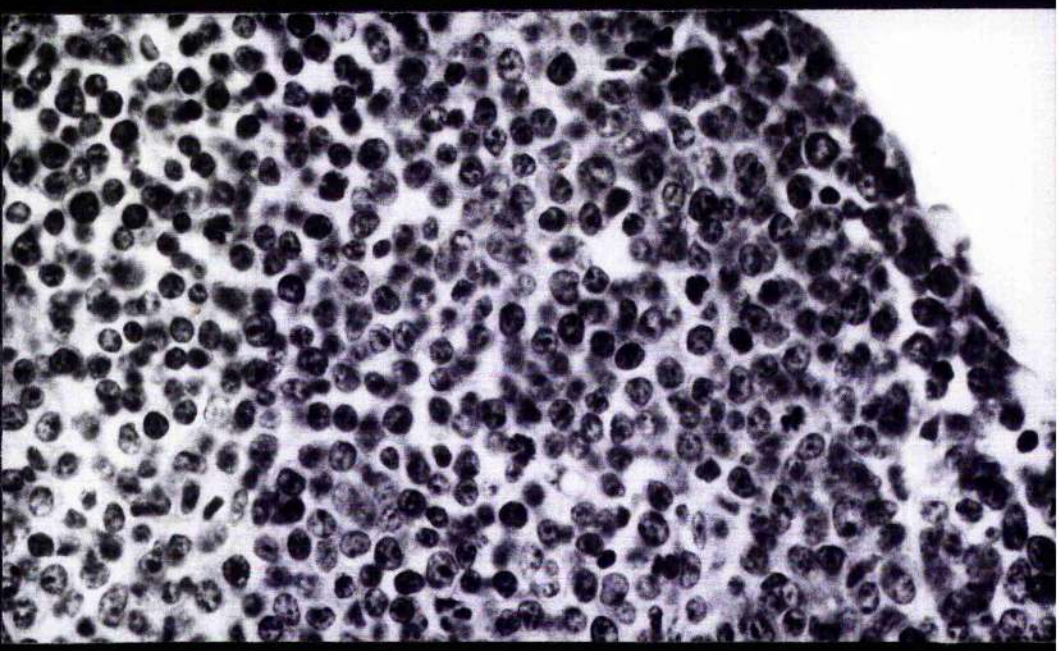
Plate (30) Higher magnification of above.

H and E x 480

Plate (31) Cytospin preparation of lymphoid cells from above.

J and G x 1200





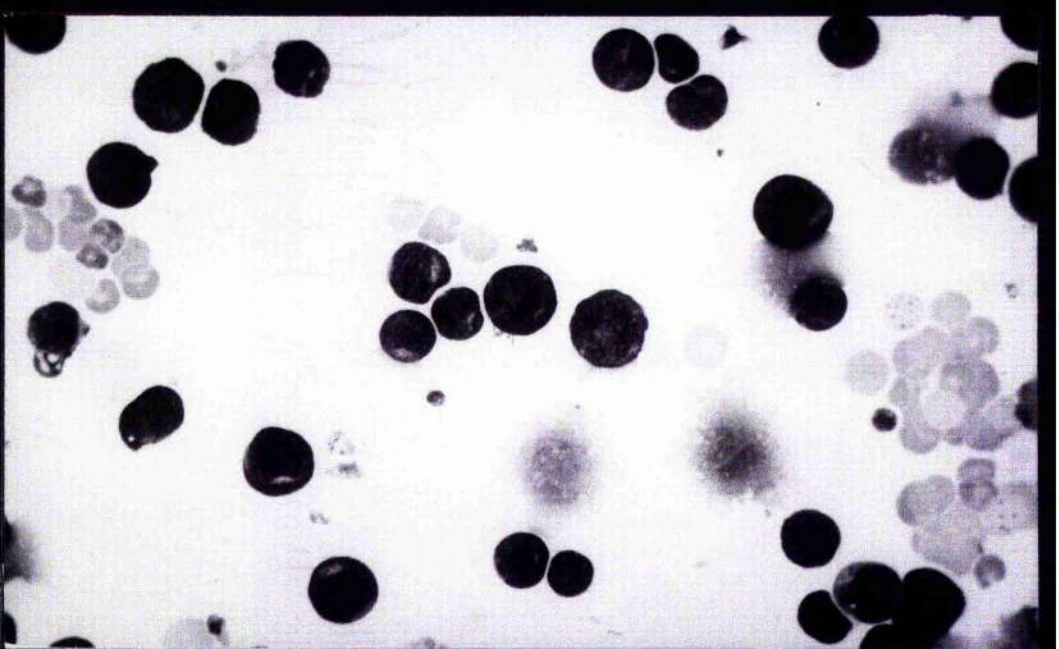


Plate (32) Thymus from 17 day mouse embryo.

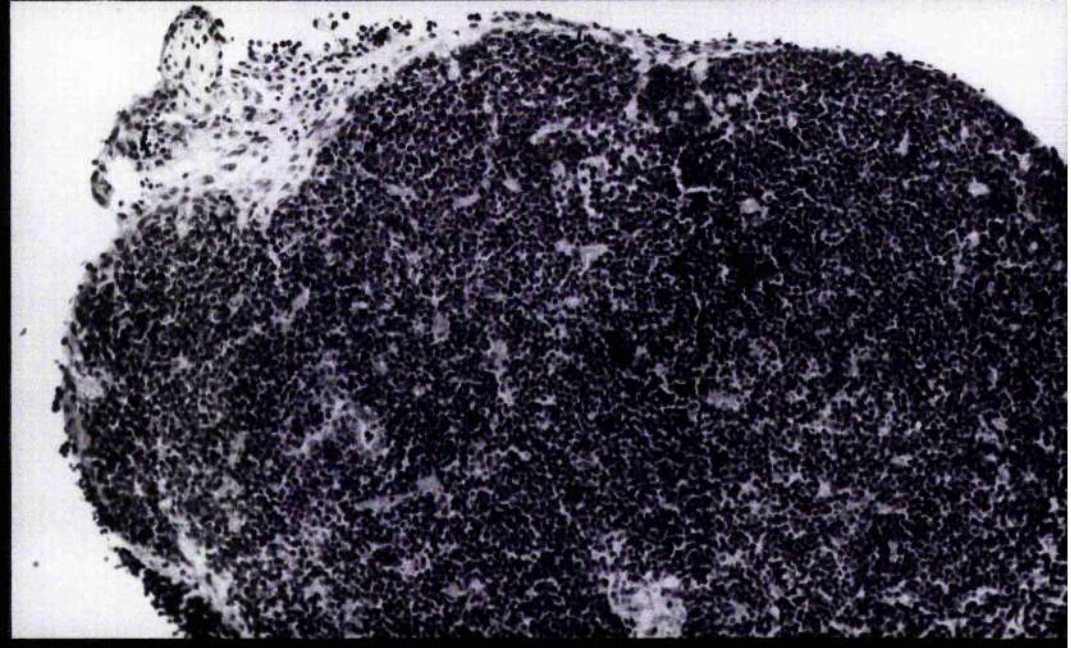
H and E x 120

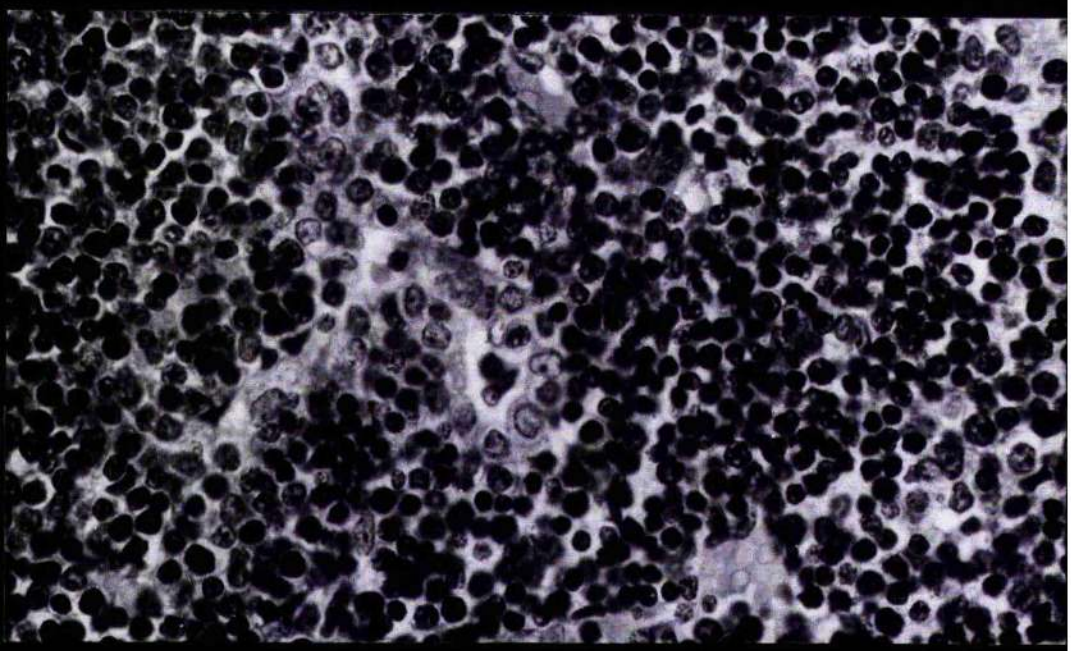
Plate (33) Higher magnification of above.

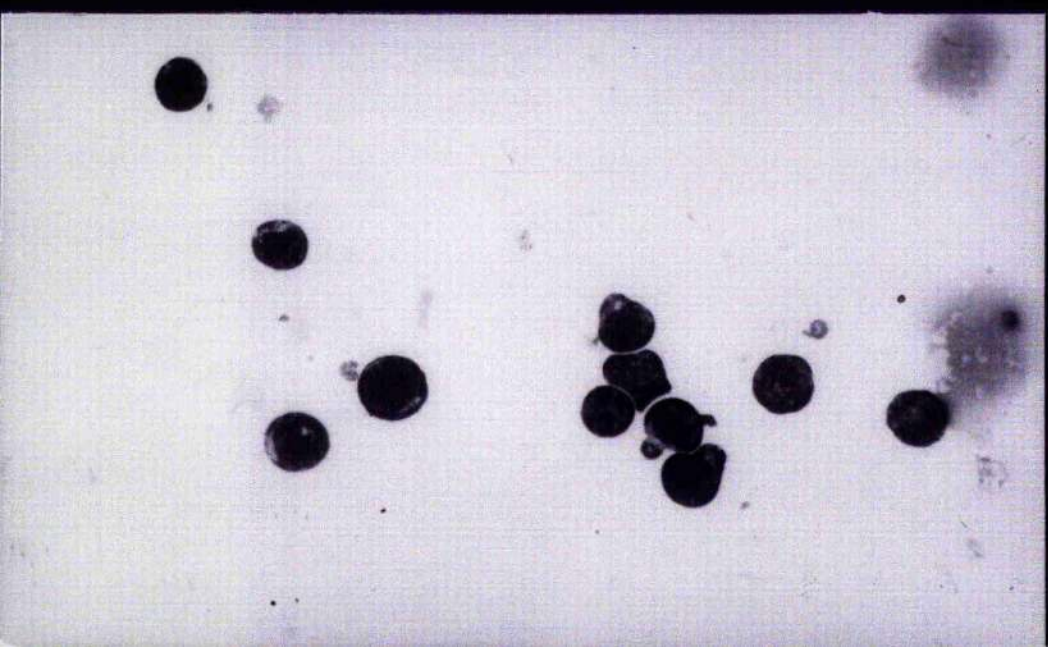
H and E x 480

Plate (34) Cytospin preparation of lymphoid cells from above, some of which are more typical of small lymphocytes.

J and G x 1200







(Plates 24, 28, 31 and 34). Large cells predominate in these preparations at days 14 and 15 and are presumably lymphoblasts. Smaller cells are more numerous at 16 and 17 days and some have the "hand-mirror" appearance more typical of small lymphocytes. While the foetal organ exponentially increases its lymphoid population between 14-18 days (M.H. Carr, personal communication), the entire organ increases rapidly in size during that time. Thus effectively the density of lymphoid cells does not increase as dramatically as would be expected from a kinetic study during that time. As the lymphoid cells are relatively sparse up till the 16th day of gestation, it was decided to use 15 day foetal thymus for the experimental protocols.

Lymphoid development in culture

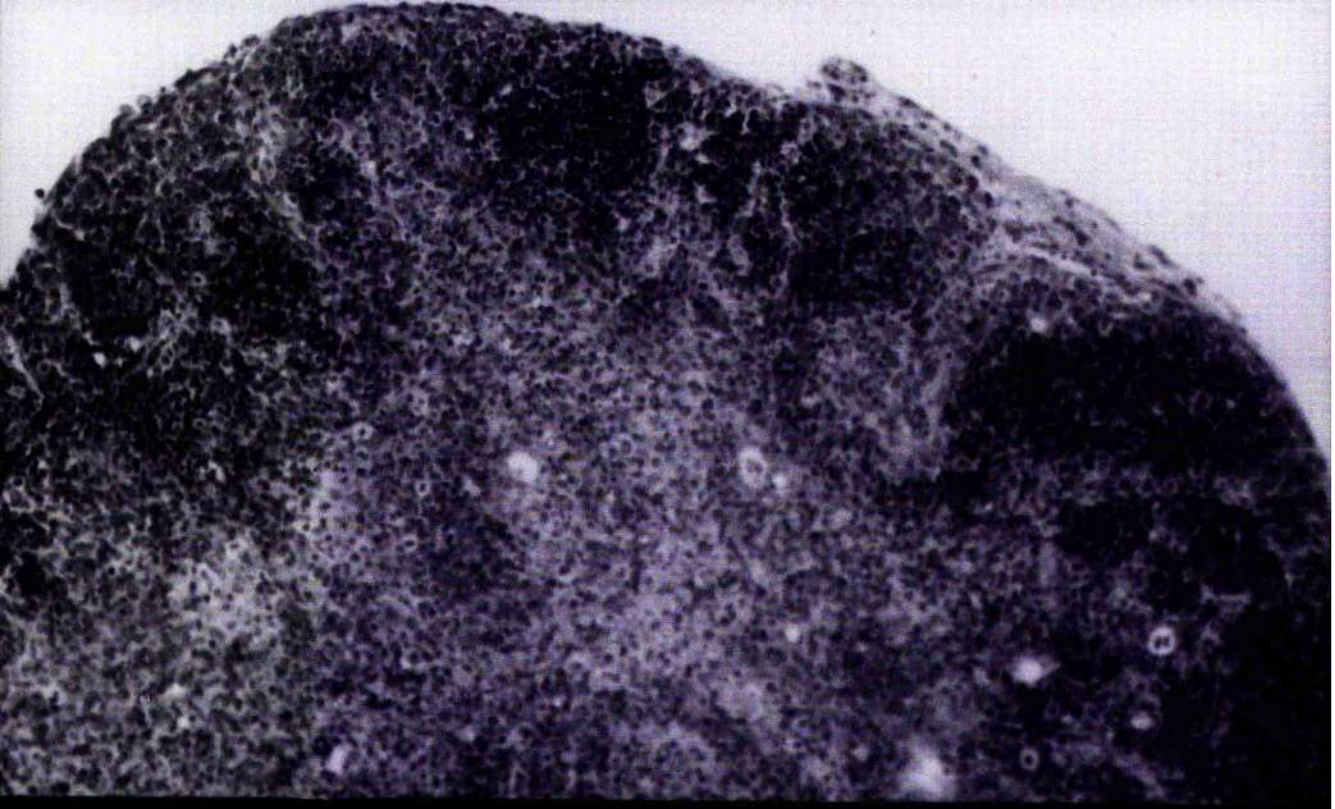
Plate (35) is a low-power photomicrograph of an explant cultured under lymphoid growth-promoting conditions for 4 days. As seen in this section, lymphoid development often occurred more preferentially at the subcapsular periphery, giving the appearance of a more densely lymphoid "cortex" and a relatively acellular "medulla". A "lymphoid medulla" alone was not seen in any of the cultures examined, it is perhaps significant that mitotic cells were seen more frequently in the "cortex" and not in the "medulla ". Plate (36) is a photomicrograph of the junctional area of the "cortex/medulla". Pale-staining epithelial cells are more numerous at the right of the picture and more darkly-staining basophilic lymphoid cells on the left, with a few cells in mitoses. Plate (37) is an electronmicrograph of an explant from a similar culture. Lymphoid cell nuclei are more electron dense, particularly

Plate (35) 15 day foetal thymus cultured to promote lymphoid growth. Appearance of a denser "cortex" and sparsely lymphoid "medulla".

H and E x 120

Plate (36) Higher magnification of junctional area of "cortex/medulla" from above. The lymphoid area on the left contains a few mitotic cells.

H and E x 480



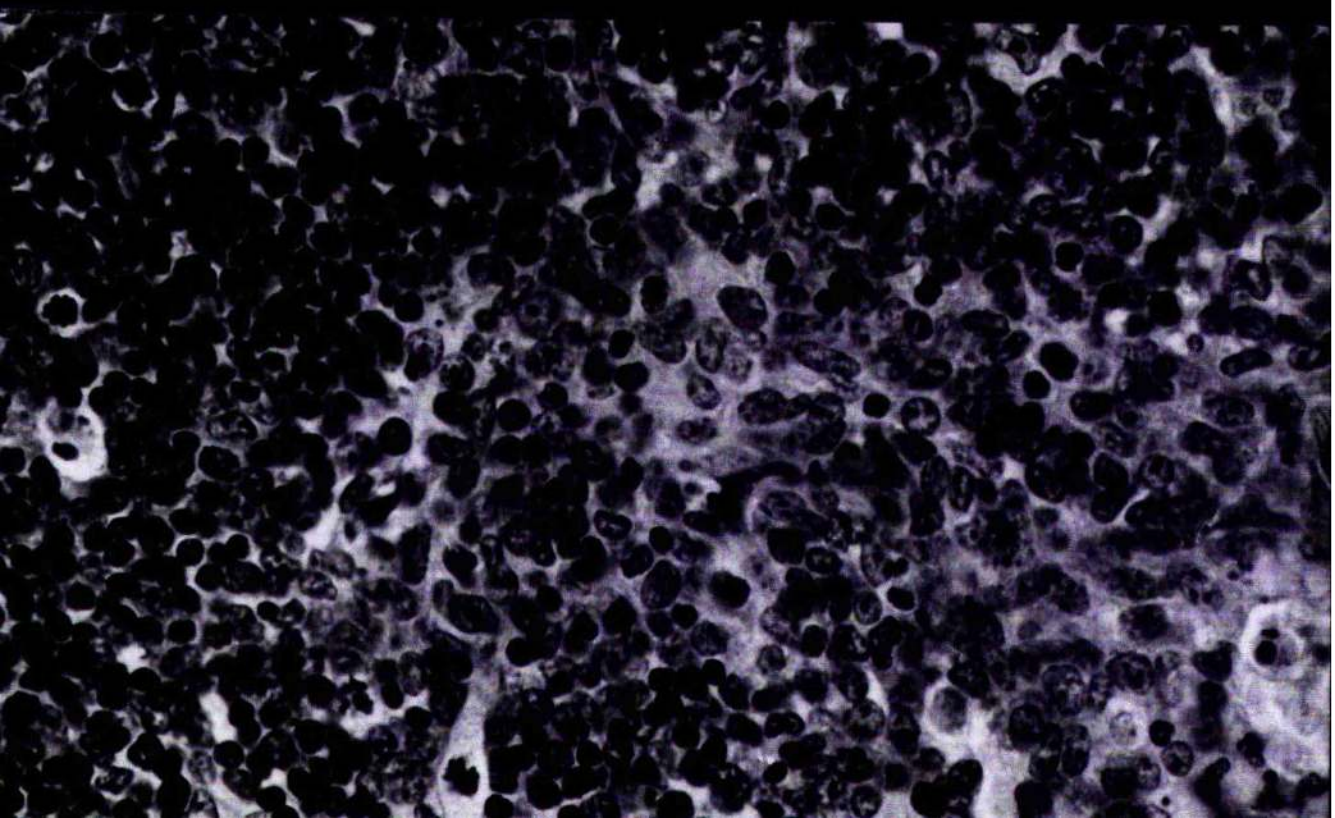
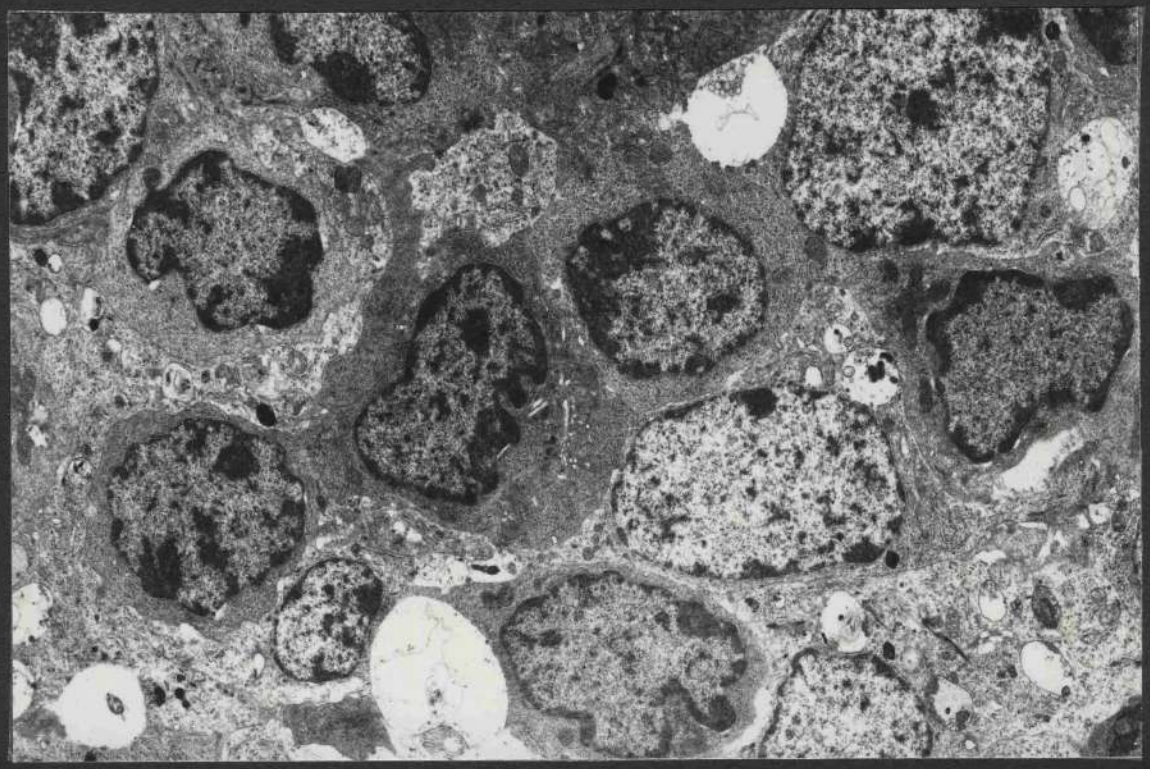
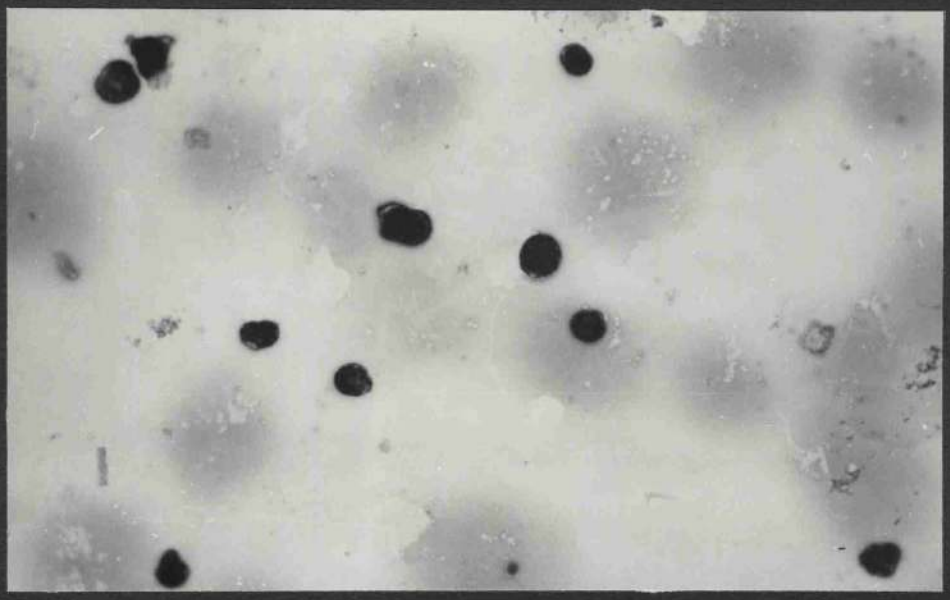


Plate (37) Electronmicrograph of an explant cultured for 4 days under lymphoid growth promoting conditions. The lymphoid cell nuclei can be distinguished by their electron-dense peripheral heterochromatin. x 6500

Plate (38) Cytospin preparation from an explant cultured as above. The cells are smaller and have the morphology of small lymphocytes, unlike the larger lymphoblasts from uncultured explants. J and G x 480





in the peripheral heterochromatin. Plate (38) is a cytocentrifuge preparation of cells teased from such a cultured explant. The cells are generally smaller than the large basophilic stem cells prepared from untreated 14 and 15 day foetal thymic tissue (Plates 24 and 28),. and the smaller cells resemble lymphocytes. The culture conditions are apparently capable of sustaining lymphoid growth, as mitotic cells are seen in this preparation.

Lymphoid depletion in culture

Plate (39) shows a section from an explant cultured under conditions inhibitory to lymphoid growth for 4 days. The tissue is essentially depleted of lymphocytes, whereas the reticulum cells have apparently survived the culture conditions. The few pyknotic cells seen are probably those of dead lymphoid cells. A cell in the telophase stage of mitosis is presumed to be an epithelial cell in division. Plate (40) is a higher magnification of the same section.

When the cultured explants were transferred to flasks and cultured so as to obtain monolayers (Chapter 1), no cellular outgrowths were obtained in any of the several attempts.

Composite foetal thymus by fusion in culture

Attempts were made to reconstitute the lymphoid depleted explants by injecting populations of haemopoietic cells; in most cases, the trauma caused to the explants rendered it unsuitable for further culture. The minute size of the explants severely limited their experimental manipulation. The problem of size was overcome by obtaining larger composite explants by fusing

Plate (39) 15 day foetal thymus cultured to deplete endogenous lymphoid cells for 4 days. The explant is predominantly epithelial, showing such a cell in division.

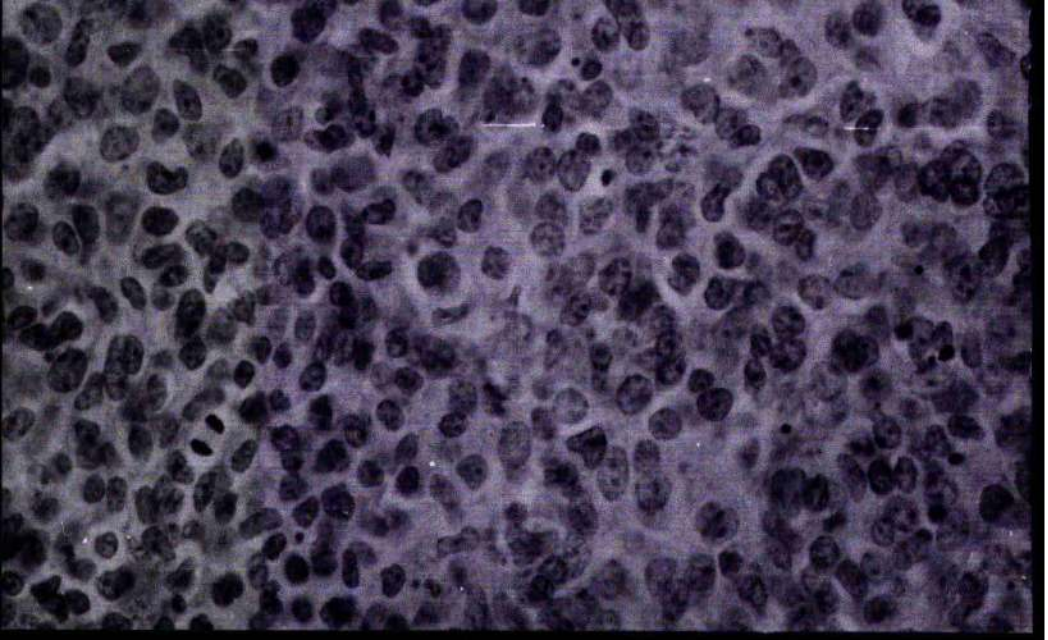
H and E x 480

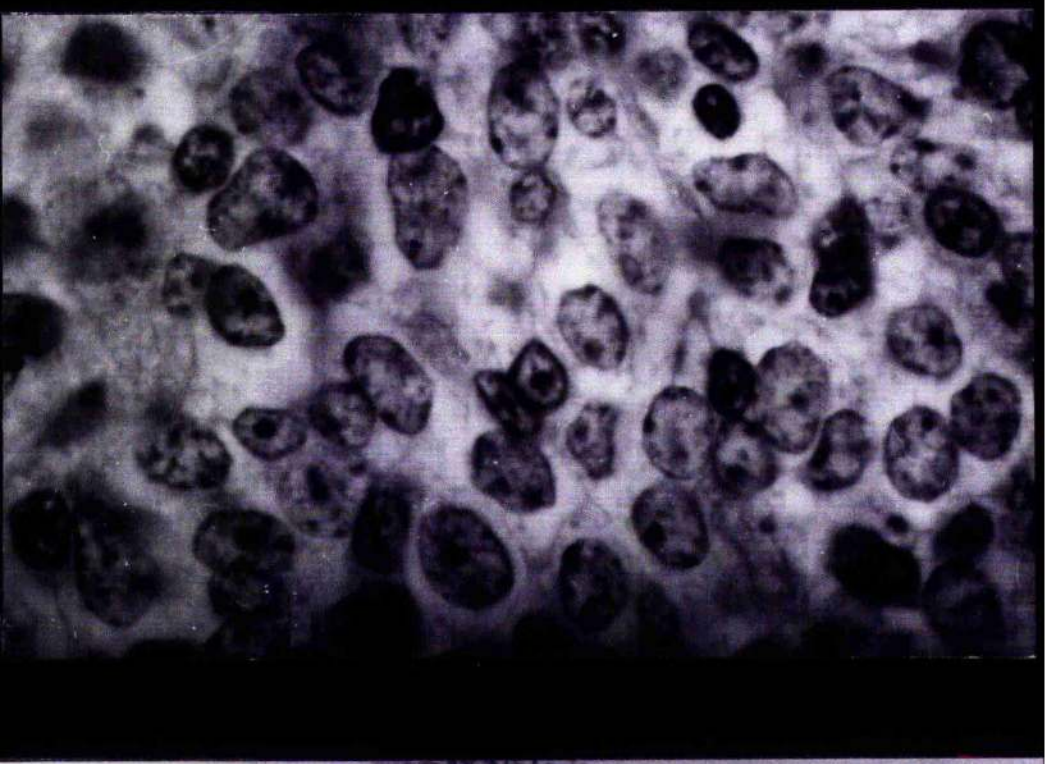
Plate (40) Higher magnification of above.

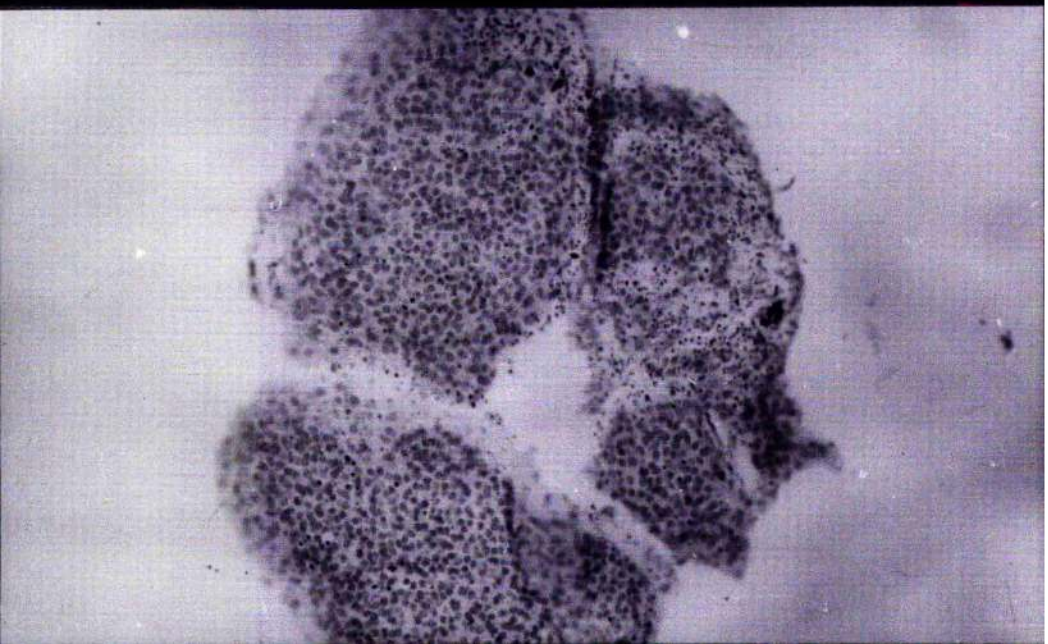
H and E x 1200

Plate (41) A composite explant composed of three 15 day explants, fused and cultured to deplete lymphoid cells for 3 days. The pyknotic remains of lymphoid cells are seen among the surviving epithelium.

H and E x 120







together individual 15 day foetal thymic lobes in vitro.

When two or more explants were co-cultured juxtaposed to each other for approximately two days, the individual connective tissue capsules were found to coalesce together to form a much larger composite explant.. Lymphoid growth or depletion proceeded under the appropriate culture conditions. 8-10 Individual explants could be fused together and subsequently cultured; but the very large composite explants were found to survive poorly in culture, with central necrosis often occurring. As a workable compromise, no more than 4-6 explants were usually fused in culture.

Plate (41) shows 3 explants fused in culture for 3 days under lymphoid depleting conditions. The individual lobes are firmly attached to each other by a coalesence of their capsular tissue. Pyknotic cells are seen amidst the surviving epithelial cells and are probably dead lymphoid cells. In later cultures, pyknotic cells appeared confined to the junctional areas between fused explants (Plate 42). To investifate whether dead cells were lost by extrusion out of the explant, the media from these cultures were collected when changed and cytocentrifuge preparations made. Nothing resembling living or dead cells were seen, although darkly stained clumped material that could possibly be cellular debris were seen. Plates (43) and (44) show a section from such a fused, depleted composite explant cultured for 6 days. It can be seen that the epithelial cells have survived and there is a scarcity of lymphoid cells. Plate (45) is an electronmicrograph prepared from a similar composite explant, showing part of the cytoplasm of an epithelial cell. The electron-dense material scattered throughout are probably gycogen granules and measure approx. 150 microns

Plate (42) Accumulation of pyknotic cells in junctional areas of fused explants, cultured for 3 days to deplete lymphoid cells.

H and E x 480

Plate (43) Fused composite explant cultured as above for 6 days. The explant appears essentially devoid of lymphoid cells while the epithelial cells have survived in culture.

H and E x 480

Plate (44) Higher magnification of above.

H and E x 1200

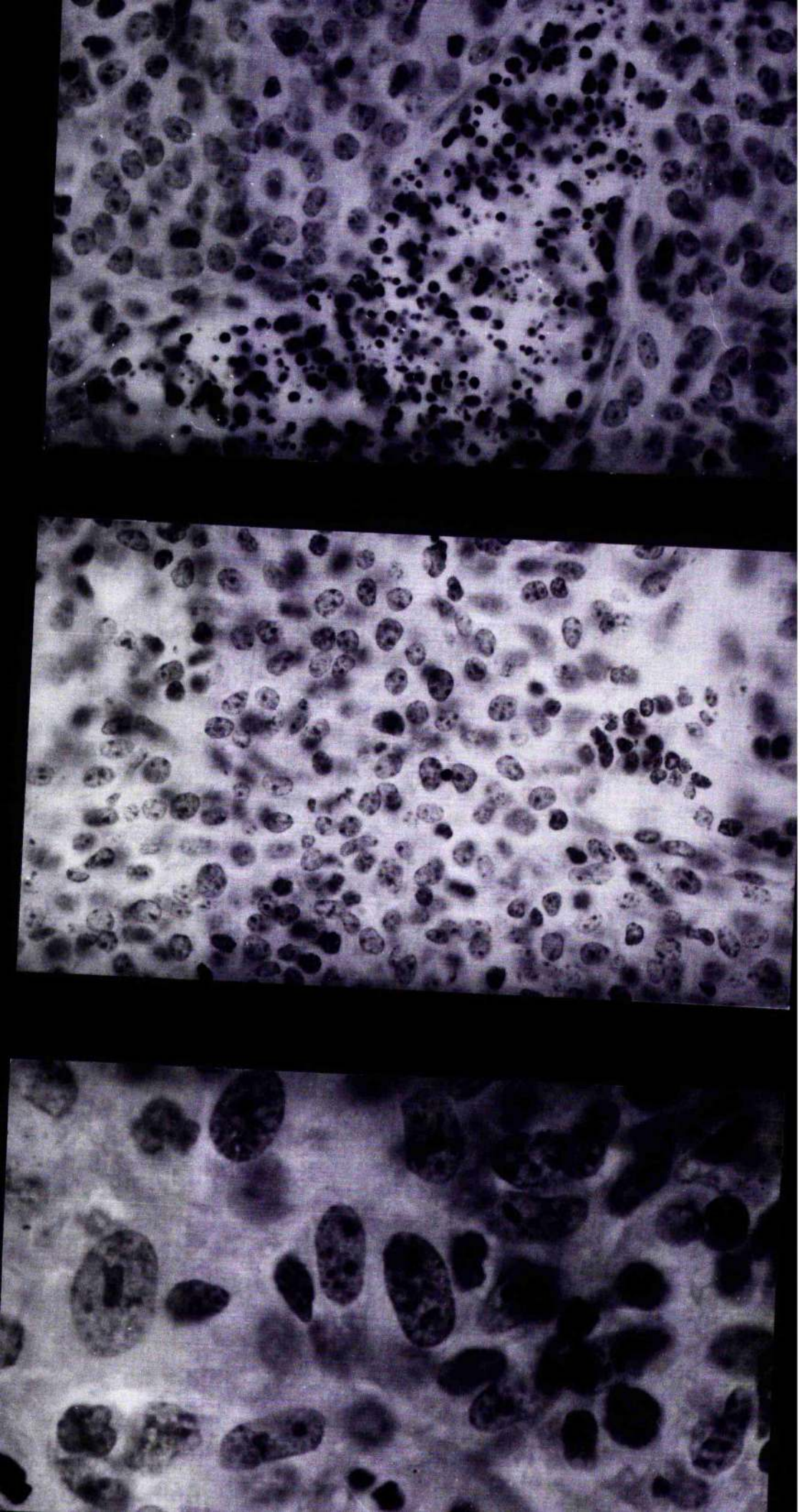
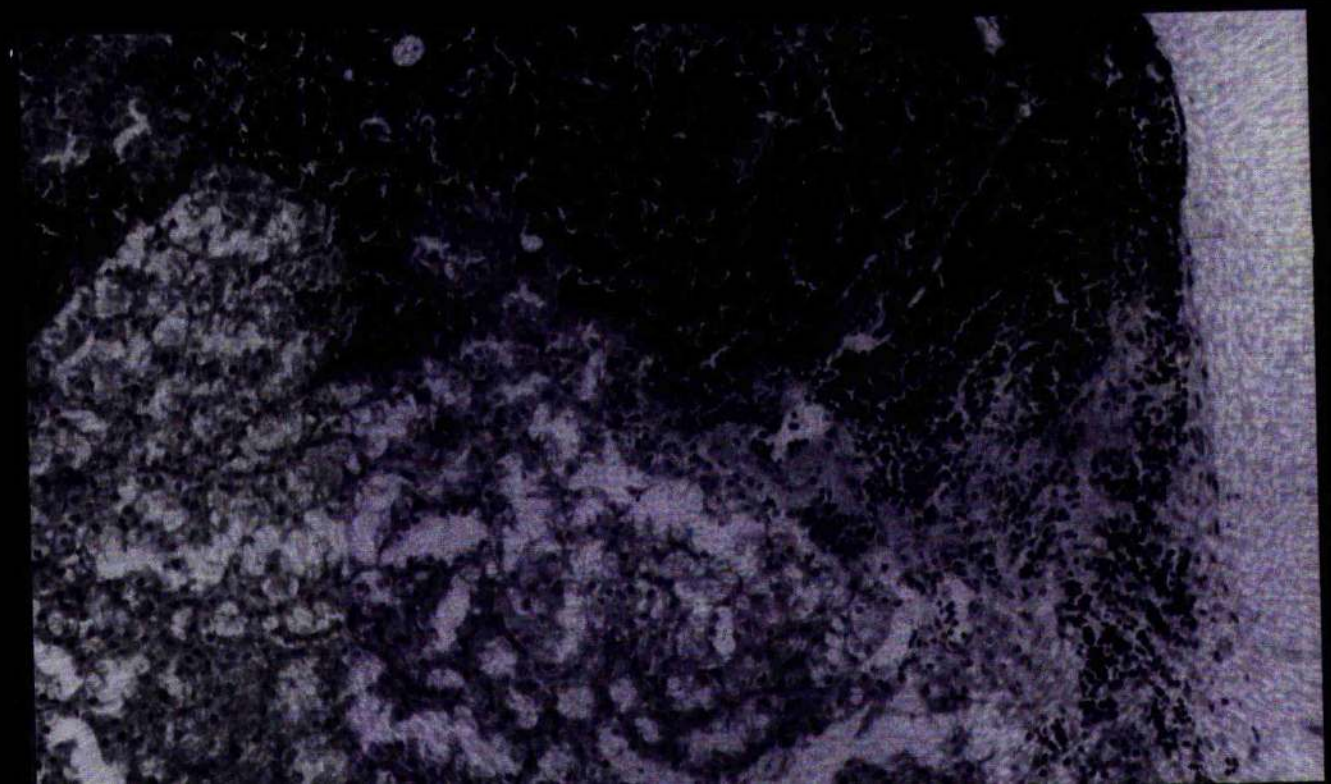


Plate (45) Electronmicrograph prepared from a fused, composite explant cultured under lymphoid depleting conditions for 6 days. The cytoplasm of an epithelial cell is seen containing what are presumed to be glycogen granules and possibly a junctional complex (arrowed)

Plate (46) An explant previously cultured for 8 days to deplete its lymphoid cells transplanted under a renal capsule. Extensive lymphoid repopulation in vivo is evident.
H and E x 120



in diameter. It is not known whether the structure on the left of the picture (arrowed) is a junctional complex or an interdigitation of adjacent cell membranes.

As these cultured explants were used to investigate the thymus repopulating capacity of different cell populations in vitro, their functional ability to be repopulated by blood-borne stem cells in vivo was investigated. Both individual and composite cultured explants previously depleted of lymphoid cells in culture were transplanted under the renal capsules of syngeneic mice. Plates (46) and (47) show a section of a kidney containing a subcapsular ectopic thymus previously cultured for 8 days, one month after transplantation. Extensive lymphoid repopulation is evident and the tissue appears healthy. Plate (48) is a section of a composite explant comprised of 4 fused thymus lobes, previously cultured under lymphoid depleting conditions for 8 days prior to transplantation. Lymphoid repopulation has occurred and a clear demarcation between thymic and renal tissue is seen. A paler, relatively acellular area in the midst of the darkly stained dense lymphoid area is seen; similar structures occur in other cultured explants and their significance is discussed later. Plate (49) is a higher magnification of the same section; the majority of cells are small, darkly stained and resemble small lymphocytes.

While examining sections of transplanted thymus, loose aggregations of lymphoid cells were occasionally observed at some distance away from the site of the transplant, in the substance of the kidney (Plate 50). Approx. 25% of the recovered kidneys showed these structures upon histological examination.

Plate (47) Higher magnification of previous section.

H and E x 480

Plate (48) Fused composite explant previously depleted of lymphoid cells in culture for 8 days, prior to transplantation under renal capsule. The in vivo repopulated lymphoid explant can be readily differentiated from the renal tissue.

H and E x 120

Plate (49) Higher magnification of above.

H and E x 1200

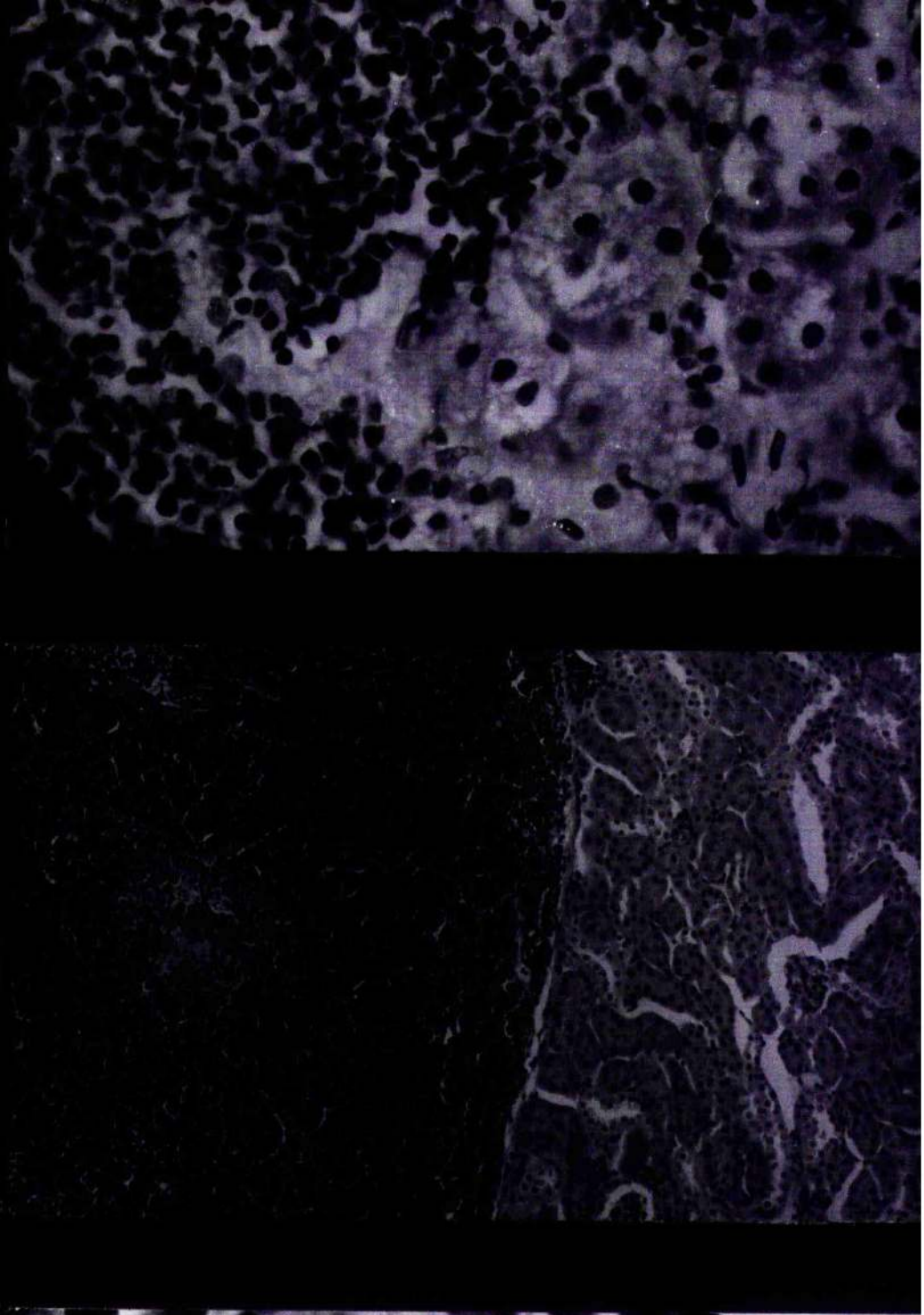


Plate (50) Diffuse nodules of lymphoid cells within substance of kidney containing ectopic thymus.

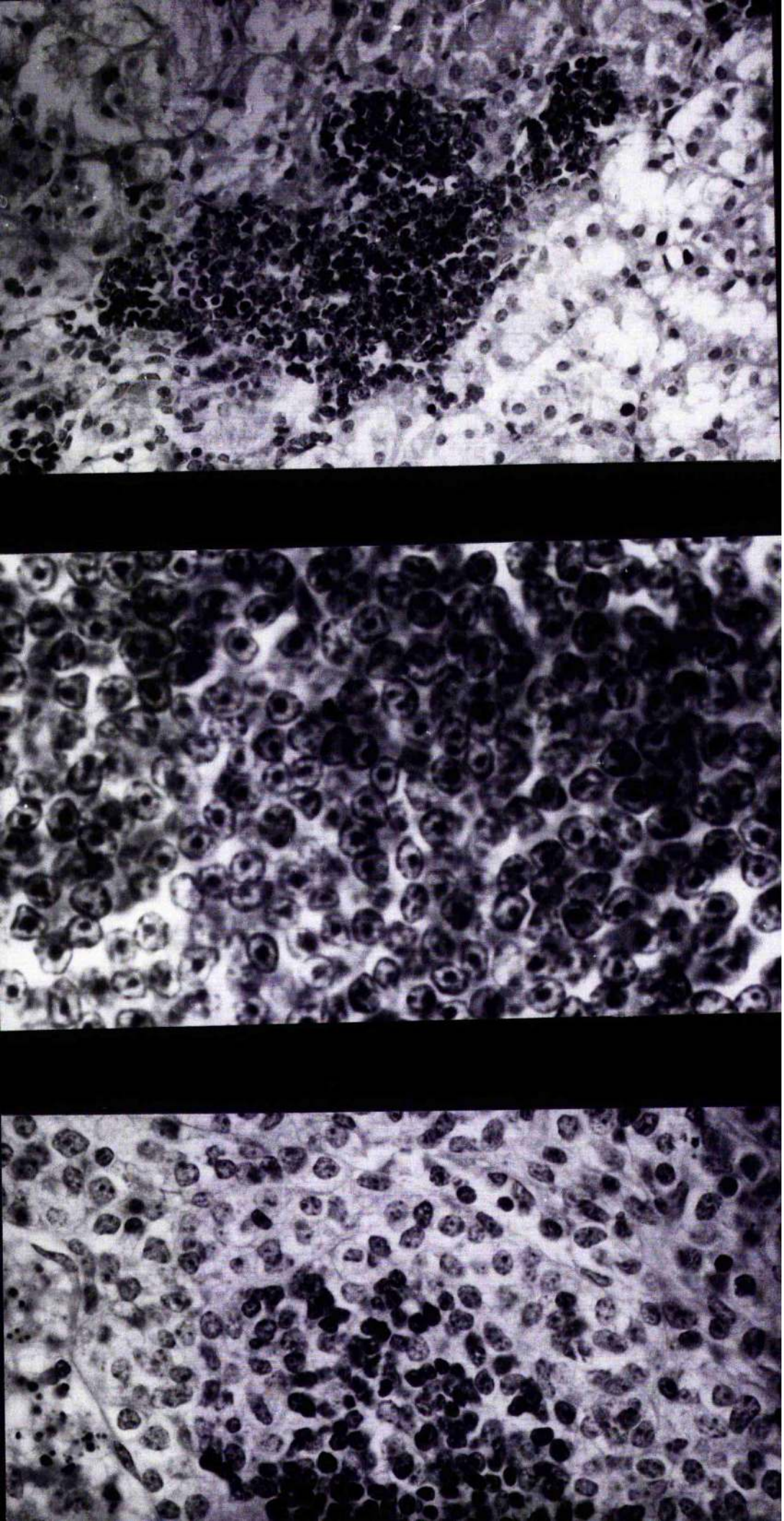
H and E x 300

Plate (51) Higher magnification of above.

H and E x 1200

Plate (52) Cultured composite epithelial explant composed of six fused lobes; injected with syngeneic foetal thymocytes and cultured to promote lymphoid growth for 2 days.

H and E x 480



Reconstitution of lymphoid depleted thymus

Populations of haemopoietic cells were injected into composite lymphoid depleted explants and cultured further to promote lymphoid development. Normal 15 day foetal thymocytes when injected into the composite explants, gave variable results; few of these were repopulated to some degree. Plate (52) shows a composite explant comprised of 6 fused lobes cultured for 2 days after injection of thymocytes. Plates (53) and (54) show a similarly treated explant 6 days after injection. The tissue is sparsely lymphoid and prolonged culture periods resulted in the degeneration of the tissue. The successful repopulation of depleted explants by an initial inoculum of nucleated cells from the bone marrow of HN_2 treated mice was not achieved. Several attempts were carried out and only pyknotic cells were seen upon histological examination after periods of culture. When such explants were injected with bone marrow lymphocytes isolated on sucrose density gradients, repopulation was largely unsuccessful. Occasionally, diffusely scattered lymphoid cells were seen when a composite explant was injected with these cells. Plates (55) and (56) show a section prepared from an explant cultured for 4 days after injection. Cells with lymphoid morphology are very sparsely distributed.

Lymphoid precursors were introduced into epithelial composite explants by their fusion with younger untreated thymic rudiments. Plate (57) shows a 14 day thymus explant co-cultured for 2 days with a composite explant previously cultured to deplete its lymphoid cells. Plates (58) and (59) are from a section made from such an

Plate (53) A reconstituted composite explant cultured as before for 6 days. The tissue is sparsely lymphoid.

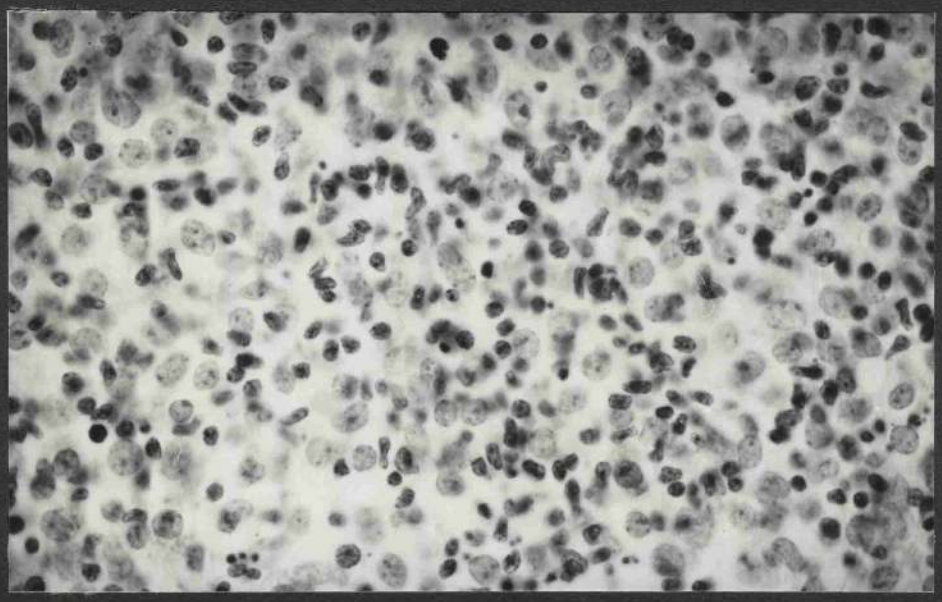
H and E x 480

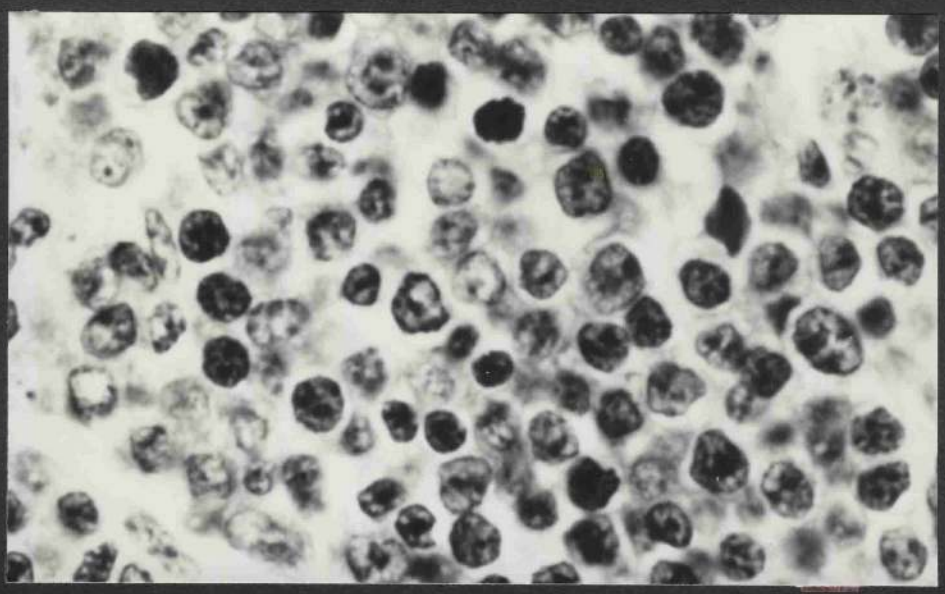
Plate (54) Higher magnification of above.

H and E x 1200

Plate (55) A composite epithelial explants injected with bone marrow cells and cultured for 4 days to promote lymphoid growth. The explant does not appear to be repopulated to any great extent.

H and E x 480





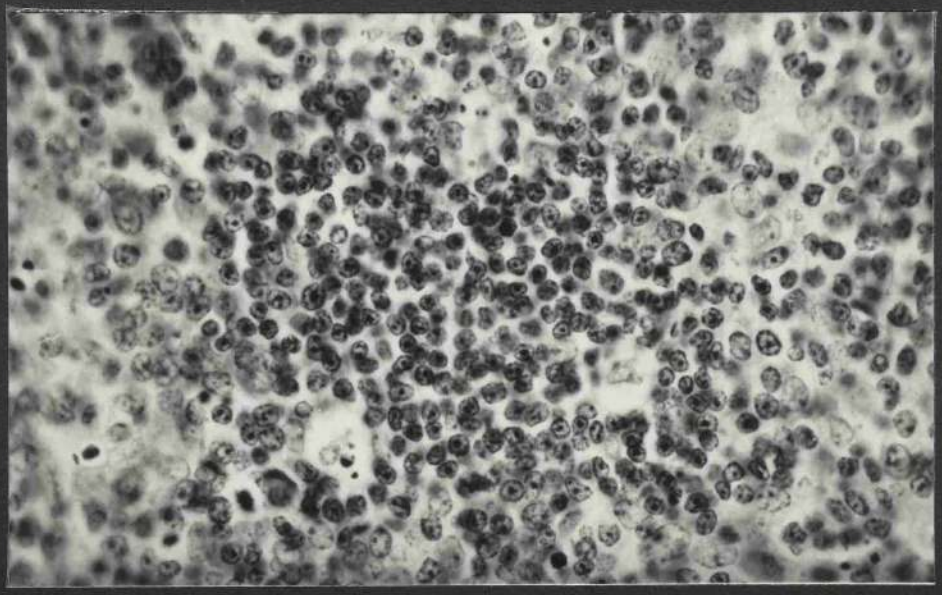


Plate (56) Higher magnification of previous section.

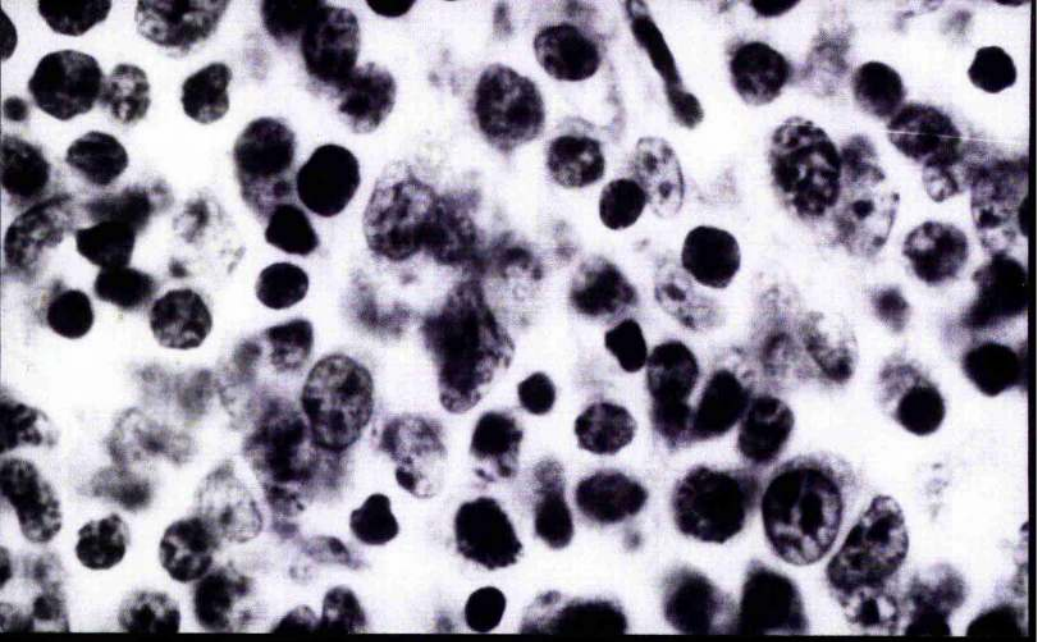
H and E x 1200

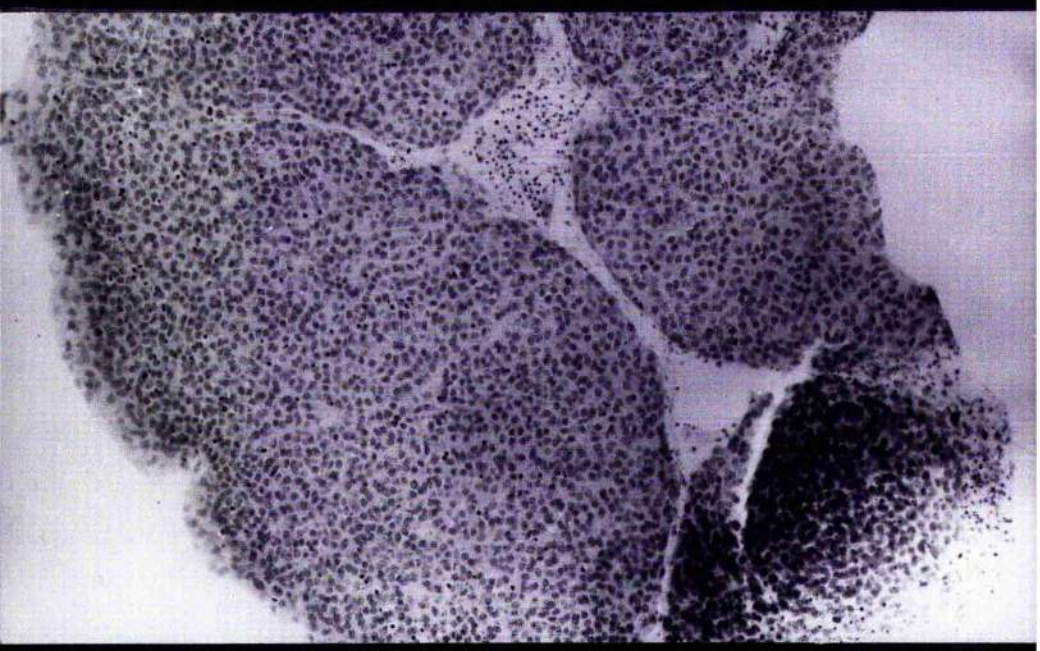
Plate (57) Lymphoid depleted composite explant fused with an untreated 14 day foetal thymus explant after co-culture for 2 days.

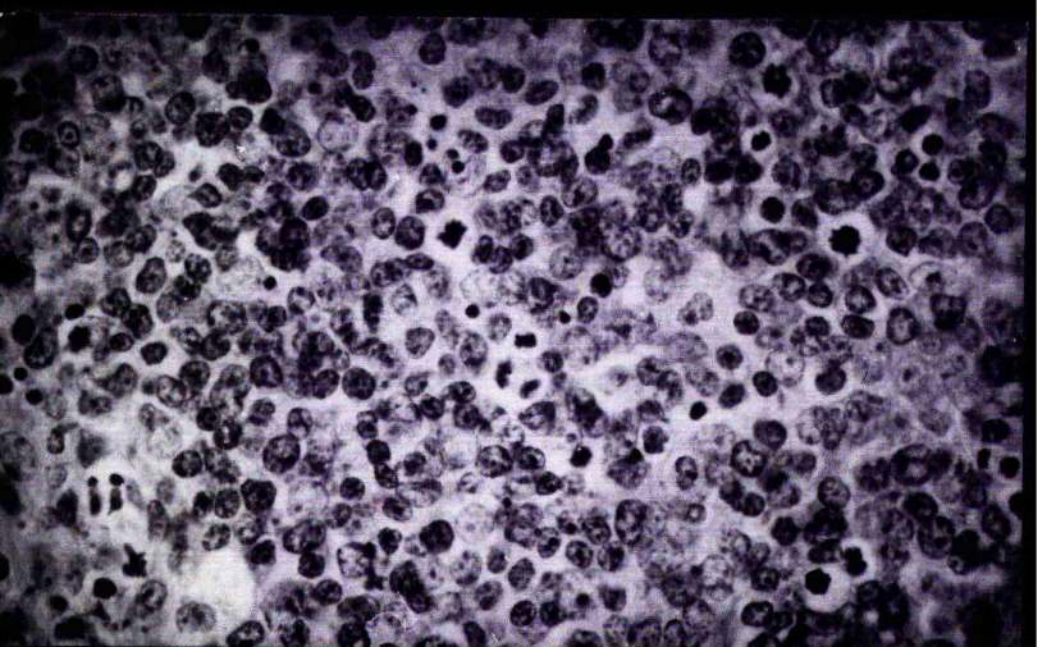
H and E x 120

Plate (58) As above, but cultured for 6 days after fusion with 14 day foetal thymus.

H and E x 480







explant cultured for 6 days. Some cells in mitosis are seen and there are a number of pyknotic cells. Plate (60) is an electron-micrograph prepared from the same culture. Two lymphoid cells and possibly a macrophage are seen in this section. Plate (61) is a section made from the same experiment, but cultured for 8 days after fusion. Lymphoid cells are more numerous and pyknotic cells are not as abundant as in the previous section; the junctional area of the fusion is seen in this section. In a higher magnification, the lymphoid and epithelial cells are clearly contrasted (Plate 62). Plate (63) shows a similar explant at the ultrastructural level. Two epithelial cells are seen, one with a prominent nucleolus. A few lymphoid cells and a macrophage are also seen in this electronmicrograph.

In some cultures, the appearance of clear, acellular areas containing small darkly stained or pyknotic cells were seen; these were invariably found to be in close association with very large, pale-staining non-lymphoid cells. They were more frequently seen in sections of older cultures (Plate 64). This section was prepared from a composite explant repopulated after fusion with a 14 day foetal thymus and cultured for 8 days. Plates (65) and (66) are higher magnifications taken from the same section. Their possible functional significance is discussed later.

Culture of thymus cells on agar

The system was tested for its ability to maintain cellular viability and possible lymphoid differentiation in vitro, under culture conditions similar to that used for lymphoid growth described

Plate (59) Higher magnification of previous section.

H and E x 1200

Plate (60) Electronmicrograph prepared from a similarly treated cultured explant as above, showing two lymphoid cells and a macrophage.

x 65000

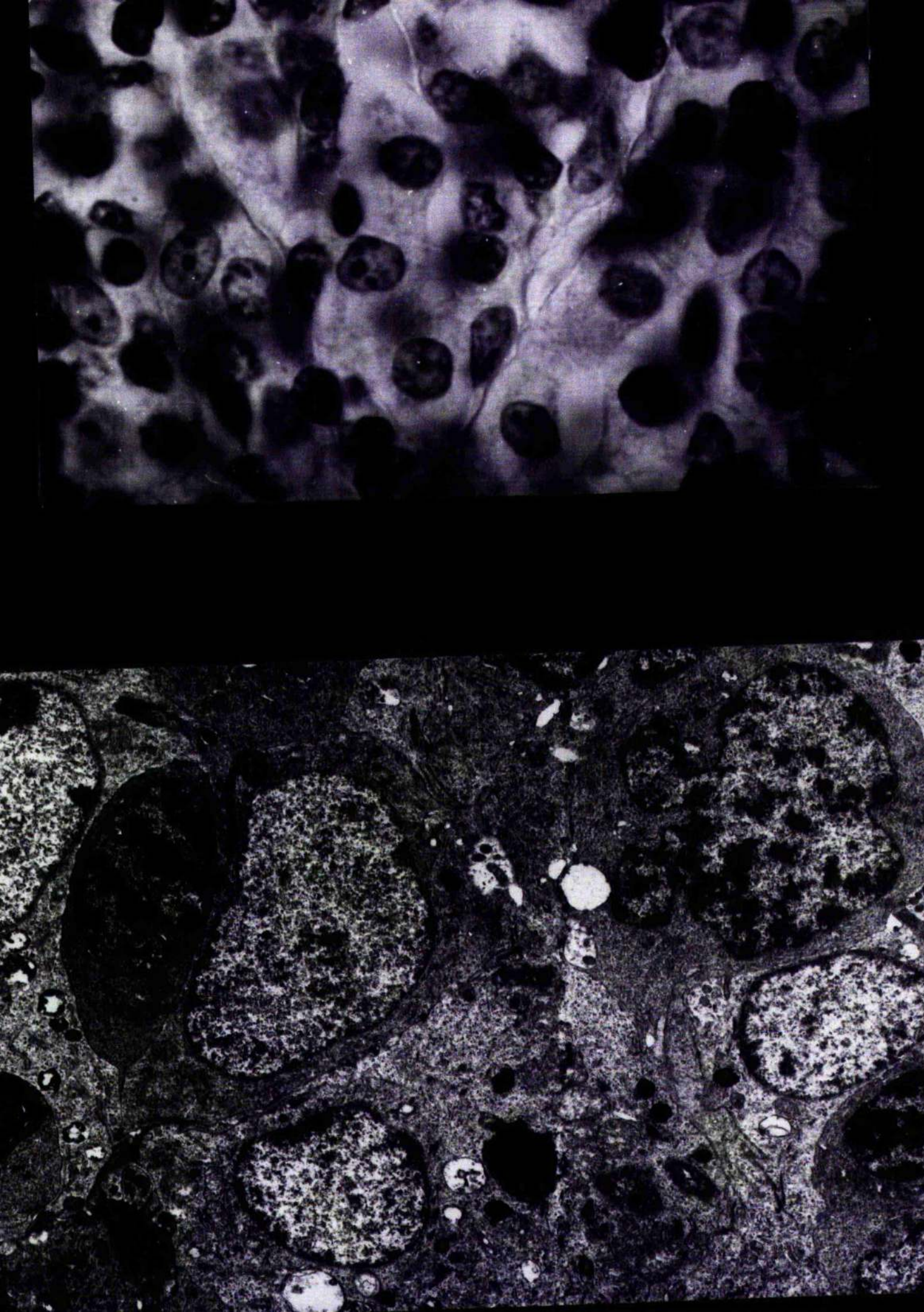


Plate (61) Composite explant reconstituted with lymphoid cells after fusion with untreated 14 day foetal thymus. Cultured for 8 days to promote lymphoid development after secondary fusion.
H and E x 480

Plate (62) Higher magnification of above.
H and E x 1200

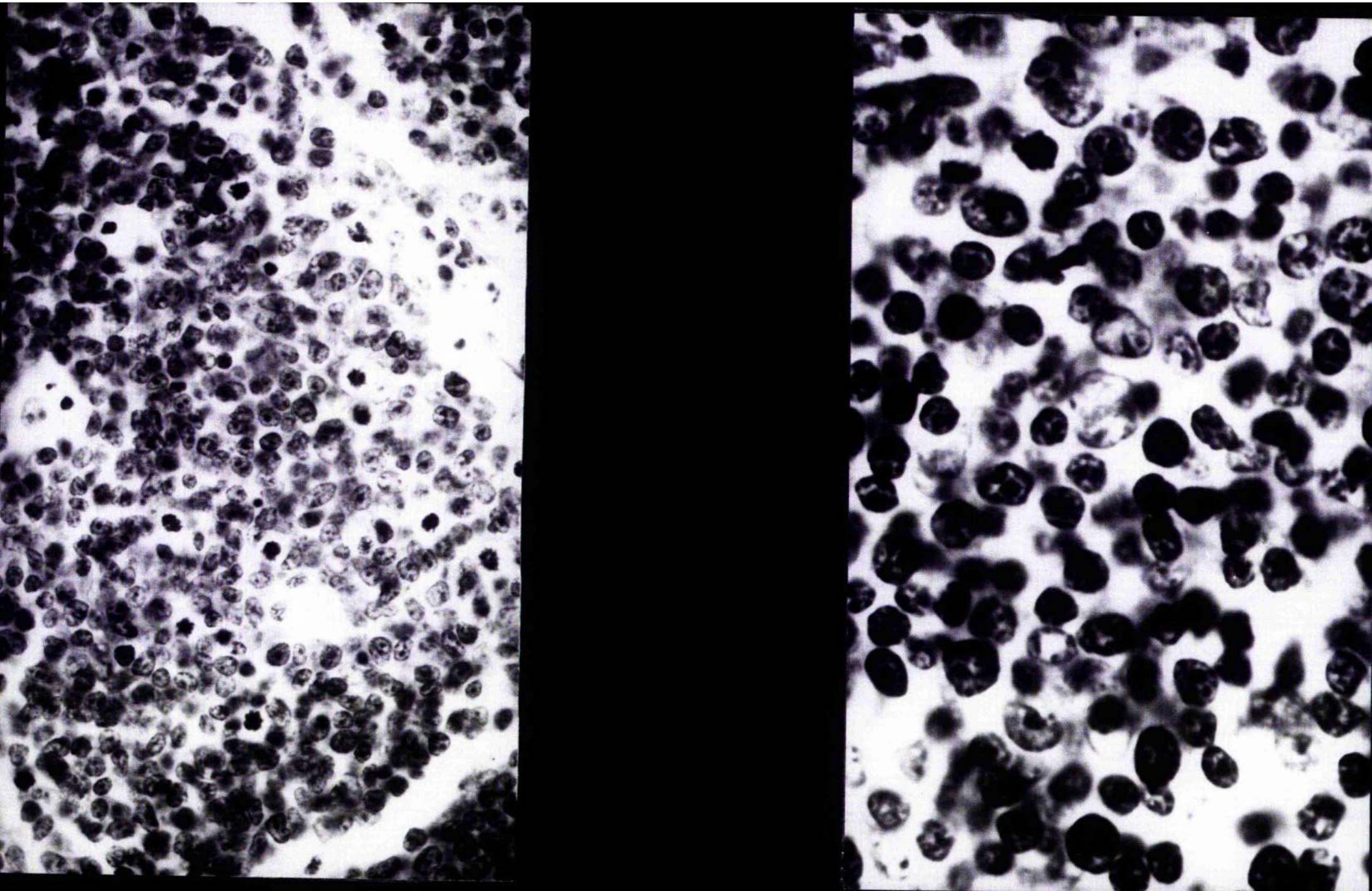
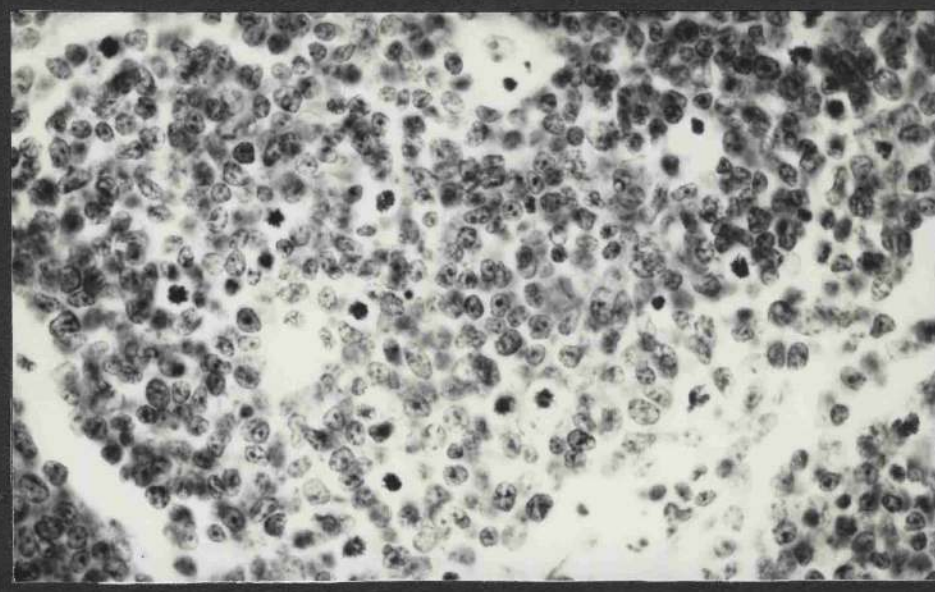


Plate (63) Electronmicrograph prepared from an explant cultured as before. One of the two epithelial cells seen has a prominent nucleolus. Lymphocytes and a macrophage are also seen in this section.

x 6500

Plate (64) Association of lymphoid and epithelial (?) cells in cultured explants.

H and E x 480



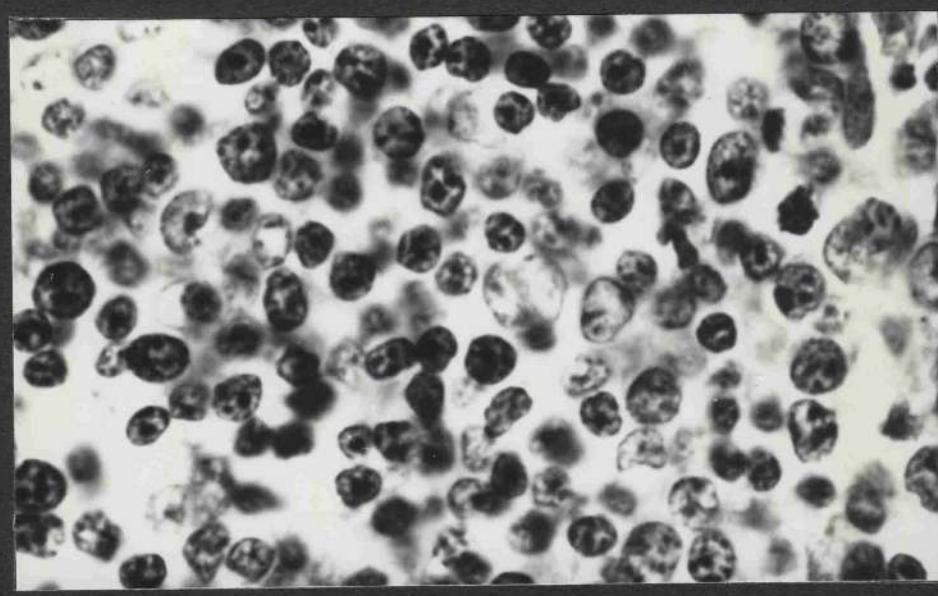
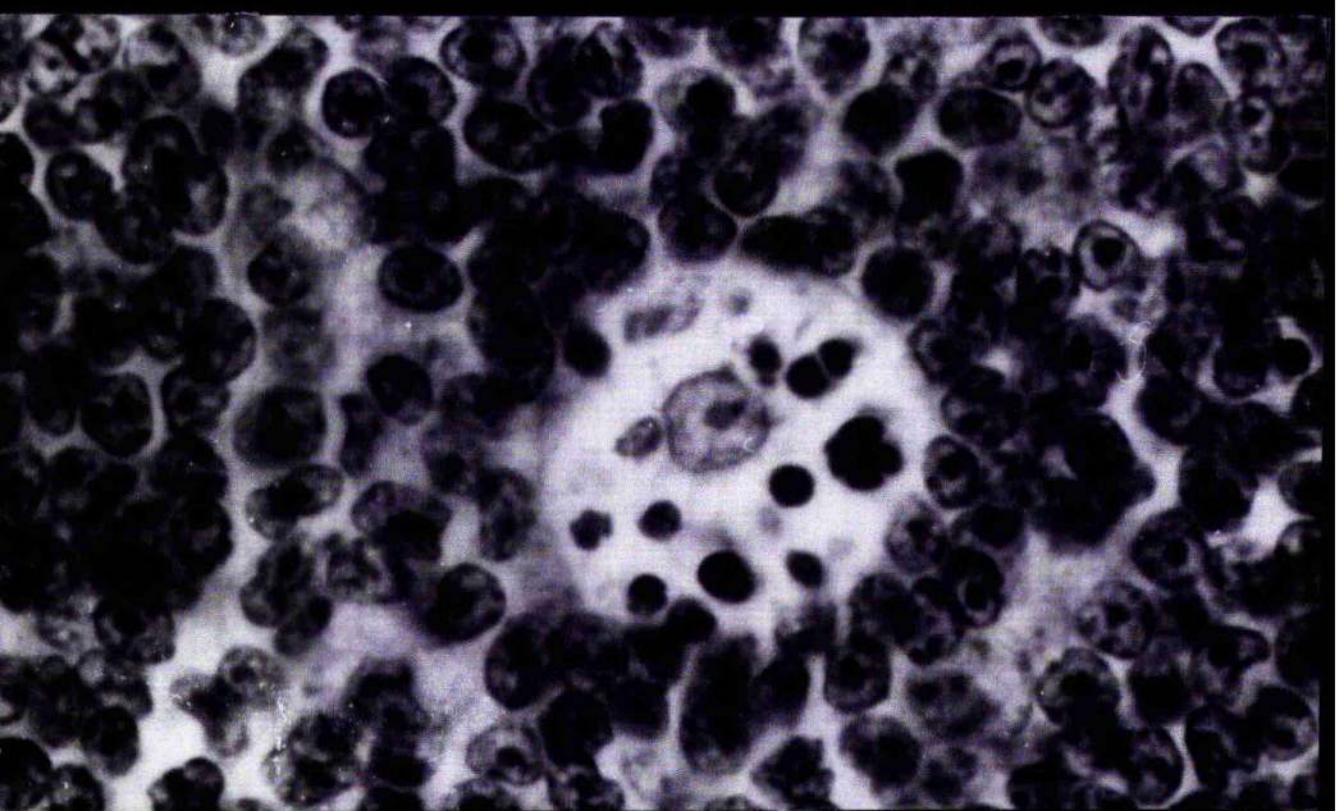


Plate (65) Higher magnification of previous section.

H and E x 1200

Plate (66) Lymphoepithelial structures from a different culture from above. Small, darkly staining lymphoid cells appear to be within the cytoplasm of the larger cell.

H and E x 1200



previously.

Plate (67) is a cytocentrifuge preparation made from an untreated trypsinised foetal thymus before culture in this system. Large basophilic stem cells are seen as well as a larger cell with paler foamy cytoplasm and an oval nucleus which most likely represents an epithelial cell. Plate (68) shows cells after 3 days in culture. Large basophilic cells are still prevalent together with smaller lymphoid cells. Plate (69) shows cells cultured after 6 days. Small darkly-staining lymphoid cells as well as larger, undifferentiated cells are seen.

Fusions and transplantation between allogeneic strains

A pilot study was undertaken where allogeneic C57/Black 14 day thymic rudiments were fused with CBA cultured explants. Generally, the explants were seen to contain largely pyknotic cells after periods of culture, poor epithelial cell survival was also evident.

Another brief pilot study was undertaken to investigate whether depletion of endogenous lymphoid cells rendered the explants non-immunogenic when transplanted to allogeneic hosts. CBA cultured explants were transplanted under C57/Black renal capsules. All of the transplanted explants were found to be rejected whereas control transplants to syngeneic mice were repopulated.

Plate (67) Cytospin preparation of cells from trypsinised 15 day foetal thymus.

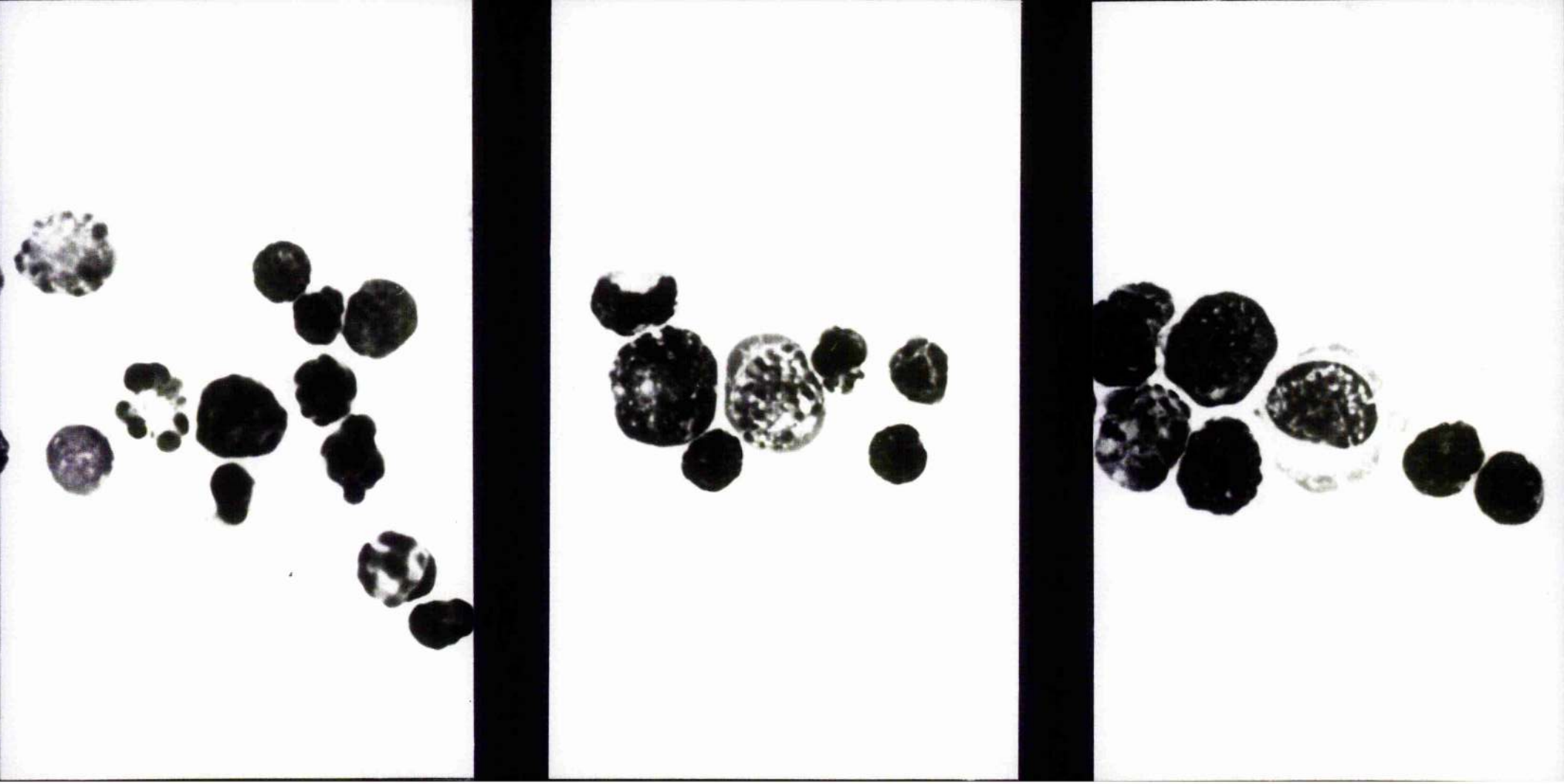
J and G x 1200

Plate (68) Cytospin preparation of cells cultured on agar for 3 days. Large, basophilic cells are still seen.

J and G x 1200

Plate (69) As above, but cultured for 6 days.

J and G x 1200



DISCUSSION

During culture to promote lymphoid growth, the appearance of a more densely lymphoid peripheral region in the explant could represent an *in vitro* correlate of the physiological organisation in the adult organ seen *in vivo*. However, it is also likely to be an artifact of culture conditions, possibly as a consequence of insufficient oxygen tension at the centre of the explant for lymphoid growth. In occasional cultures, it was noted that the explants flattened out on the millipore stratum and assumed a disc-like shape. This is thought to be a consequence of a compensatory response to inadequate oxygen tension (R.K. Jordan, personal communication). The effect of Waymouth's medium on inhibiting lymphoid growth is not entirely convincing, as usually explants cultured in RPMI or Eagles medium could also be depleted providing the other parameters were adhered to. Therefore, the oxygen content of the atmosphere and the initial lack of serum supplement were most likely the causative factors for inhiting lymphoid development. When untreated foetal thymus was explanted in culture flasks, monolayers of macrophages were readily derived (Chapter 1). However, as macrophages were not similarly obtained from cultured explants, it is tentatively suggested that they are also selectively lost during culture. Although no cellular outgrowths of any kind were obtained from these explants when plated in culture flasks, their functional viability was found to be satisfactory as they were readily repopulated after transplantation *in vivo*.

The spurious occurrence of nodules of seemingly lymphoid tissue at regions away from the sites of renal subcapsular

transplantation is curious. It is not known whether they represent ectopic sites of secondary lymphoid tissue or an infiltration of lymphocytic cells following an inflammatory response. If the latter possibility is the case, these cells would be expected to be small lymphocytes. However, in the high power photomicrograph, they morphologically resemble lymphoblasts rather than small lymphocytes.

It was considered that the major cause for the lack of success in injecting cultured explants was that the minute size of the explant limited its manipulation without severe damage to the tissue. This problem was overcome by obtaining the larger composite explants after fusion in culture. The fusion of intact tissue fragments was first demonstrated by Sigurdsson (1942). It is not known how the dead lymphoid cells are removed from the cultured explants. They could be either phagocytosed by thymic macrophages, if present; or somehow be extruded from the substance of the explant by the reorganisation of other cells. Evidence for this latter possibility is presented by Wiseman (1977) who found that cell-size spheres of metal, glass and plastic could move from surface to subsurface positions within solid tissue masses in culture, demonstrating that the observed movement of cells in similar circumstances may not be due to active cell locomotion. These extruded pyknotic cells would thus get trapped within pockets between the coalesced capsules. As the thymic subcapsular region is normally rich in macrophages, accumulations of unphagocytosed dead cells would not be expected. This observation could be interpreted as indirect evidence that thymic macrophages are also depleted under these culture conditions.

Bone marrow cells from HN_2 treated animals were not able to repopulate the explants. However, it is not known whether this was due to the limitations of the experimental system or because of the functional immaturity of these cells. A kinetic study of the thymus repopulation of irradiated mice by such cells in vivo would resolve this question. The results obtained with density gradient enriched bone marrow lymphocytes are not conclusive. The evidence that they represent a pre-thymic population inducible in vitro to show T cell characteristics could not be corroborated in another study (Chapter 3). Upon their attempted transference into the thymic environment in vitro, they were not able to repopulate the organ, as far as can be ascertained in this system.

The rationale for fusing an uncultured 14 day foetal thymic rudiment and a temporally older cultured explant was directed by the following reasons. The embryonic thymus at 14 days of gestation is a source of undifferentiated stem cells. Furthermore, the ease with which foetal thymuses were found to coalesce in earlier experiments enabled the reconstitution of an epithelial thymus, as cells were found to migrate from the younger to older explants. Such fusion between embryonic gonadal tissue has been used by reproductive physiologists to investigate microenvironmental effects upon germ cell maturation (C.W. Evans, personal communication). Any organisation of the cellular architecture of the embryonic organ would, as a consequence of fusion, be perturbed. However, this possibility does not appear to inhibit successful repopulation of the explants, which argues against any

compartmentation within the embryonic thymus. In these explants reconstituted after fusion or injection and following periods of culture, cells morpholologically resembling small lymphocytes were not numerous, and lymphoblasts predominate. This is probably a consequence of the experimental manipulations subjected to the tissue. It was not possible to prolong the cultures for longer periods as central necrosis was often seen after 2 weeks of culture.

The culture of thymic tissue on agar blocks was based on a method used by other investigators to maintain foetal gonadal tissue in vitro (T.G. Baker, personal communication). The technique was devised to aggregate trypsinised foetal thymus tissue in agar tubes prior to their culture. The system was tested for its ability to maintain cellular growth as in the intact thymus under similar conditions. Small lymphocytes were not numerous after culture up to 6 days and mitotic cells were not evident in the preparations made from them. However, the system does maintain cellular viability for limited periods in vitro and could be adapted to investigate microenvironmental effects during co-culture of diverse cell types.

Experiments where foetal thymus from histocompatibly different strains were fused in culture were, on the whole unsuccessful. As control cultures of syngeneic fusions also deteriorated, failure could be attributed to poor culture conditions, and no conclusions can be derived from such a brief investigation.

Cultured explants when transplanted to allogeneic mice were rejected. Histological sections from similarly cultured control explants confirmed that the tissue was healthy and was repopulated

when transplanted to syngeneic hosts. Thus rejection *in vivo* must be attributed to histocompatibility differences and a failure to render the tissue unimmunogenic, rather than its inability to be repopulated by host stem cells.

The significance of vacuole-like structures in cultured explants, containing large pale staining cells with a cluster of darkly staining cells resembling small lymphocytes or pyknotic cells, either closely associated with or within the large cells can only be speculated. They are not restricted to explants *in vitro* as they still persist or occurred *de novo* when transplanted *in vivo*. It is possible that these structures represent cells in the primary stages of formation of Hassall's corpuscles; these are thought to arise by a fusion of epithelial cells. Some of these structures are seen to contain more than one nuclei which resemble those of epithelial cells. A histochemical study to detect PAS positive material would further elucidate the nature of these cells. A much more speculative alternative would be to consider them as dynamic structures involved in the differentiation of thymocytes, the smaller cells undergoing some kind of cellular interaction with the large pale staining cell during the process of maturation. The appearance of "rosettes" comprised of thymic macrophages surrounded by thymocytes during the very early stages of a monolayer culture have been observed by other investigators. It is thought that they might represent a differentiation event, from evidence with time-lapse cinematography (J.G. Sharp, personal communication). It is possible that these structures seen in this study could be similar to the giant "nurse cells" described by Wekerle and

Ketelsen (1980) in their monolayer cultures; these were found to engulf and subsequently release a large number of thymocytes, a process believed to be involved in lymphocyte differentiation. The identity of the large pale staining cell is not known, if it proves to be a macrophage, then it raises questions for further study.

CHAPTER 3

T cell precursors

INTRODUCTION

Haemopoietic cells capable of seeding the thymus have been detected in the bone marrow (Harris et al 1964). Further evidence for the presence of precursors of T cells in the marrow was obtained when bone marrow cells were found to be inducible targets for the effects of thymic humoral factors and their pharmacological analogues in vitro. There is also evidence that an immunologically competent population of thymus-derived cells transiently resides in the marrow, believed to be recirculating T cells. These cells are thought to be responsible for the GVH disease encountered after clinical bone marrow transplantation. Dicke et al (1969) have isolated this population from the bone marrow of rats and primates by centrifugation on discontinuous BSA density gradients. Burleson and Levey (1972) have similarly isolated GVH reactive cells from murine bone marrow. It is apparent that the bone marrow is also a primary site of production of lymphocytes. The origin of marrow lymphocytes and their functions is reviewed by Rosse (1976). Ig Fc and Complement (C) receptors have been detected on marrow lymphocytes (Yang and Osmond, 1979); as well as surface Ig by radiolabelled antiglobulin binding studies by Osmond and Nossall (1974a and b). These studies show that the marrow is a major site of renewal of Ig-bearing small lymphocytes. Other cells in the bone marrow have been shown to lack both surface Ig and the Thy 1 antigen, they are not thymus dependent and nor are they part of the recirculating T cell population.

It has been reported that the population of PHA responsive cells in the bone marrow are not thymus dependent (Blomgren

and Svedmyr, 1971). Furthermore, Ropke (1977a and b) has detected increased numbers of Thy 1 bearing cells in the bone marrow of NTx and nude mice, as compared with normal and sham Tx mice. Autoradiographic and kinetic studies indicate these cells are produced in the marrow itself, presumably as a compensatory response for the lack of normal thymic lymphopoiesis. The mitogen responsiveness and GVH reactivity of these cells suggest they are functionally immature. Cohen and Patterson (1975) have found that the Con A and PHA responsive population of small lymphocytes in the bone marrow could be induced to express Thy 1 and TL antigens by the mitogens as well as by cAMP and thymosin. They postulated that these cells in the bone marrow were T precursors. These cells were also able to enhance the Con A responsiveness of cortisol-resistant thymocytes synergistically (Cohen and Fairchild, 1976).

It is evident that the bone marrow lymphocytes are a heterogenous population, just as their peripheral counterparts. The only feature in common within and between both populations is their morphology. Attempts to isolate homogenous fractions from these have necessitated exploiting differences in their physical properties such as size and specific gravity. The pre-T cells in bone marrow have been isolated by density centrifugation on linear gradients of Ficoll by El-Arini and Osoba (1973) and were shown to be distinct from the CFU-S population.

Density centrifugation on gradients of sucrose have been employed by Osmond and Yoshida (1971) to isolate lymphocytes from guinea pig and rat bone marrow. A modification of their

method has been used by Press and Rosse (1977a) to fractionate the lymphocyte population of mouse bone marrow. These cells exhibited a blastogenic response upon culture with high doses of PHA for prolonged periods. Approx. 20μg/ml of PHA maximally stimulated these cells, the peak proliferative response occurring between 4-7 days of culture. The system was considered to enhance the otherwise meager PHA reactivity seen with whole, unfractionated bone marrow cells by about 15 fold. The distribution profile of these cells on the gradient was similar to that of erythrocytes and could be readily detected visually and recovered with the erythrocyte-rich fractions. It is not known whether the PHA reactivity of these cells was acquired similar to an antigen-driven maturation, leading to a proliferative response, as happens after antigen-priming of virgin lymphocytes. Alternatively, this could represent a maturational event outside the thymic environment. Investigation of the first hypothesis is outside the scope of this study. However, if the latter alternative is a tenable possibility, it would be further elucidated by prior exposure of the cells to the thymic environment, such as by co-culture with thymus-derived monolayers or by incubation with their supernatants. Any degree of immunocompetence induced in these cells might possibly alter the kinetics of their PHA response. It was intended to follow such a line of investigation.

PROCEDURE

A linear sucrose gradient generating apparatus was designed, constructed and tested for reproducibility of cell recovery. The lymphocyte fractions with the distribution profile of erythrocytes were recovered from such gradients and incubated with PHA, closely following the culture conditions of Press and Rosse (1977a) as far as possible.

Bone marrow lymphocytes isolated from such gradients were co-cultured with thymus-derived cellular monolayers to possibly induce the expression of T cell differentiation antigens (Chapter 1). They were also used in the attempted reconstitution of embryonic thymuses depleted of their endogenous lymphoid cells (Chapter 2).

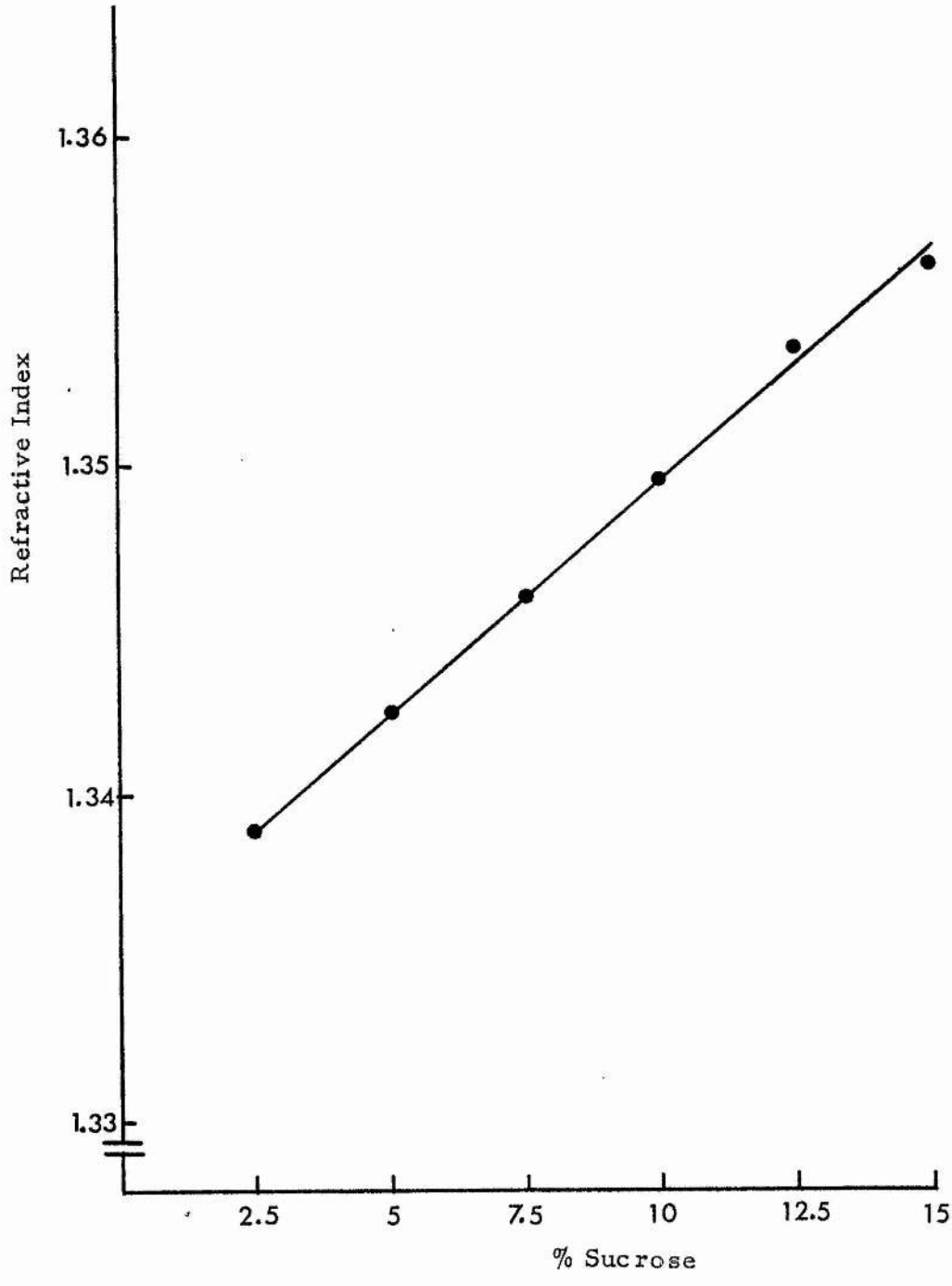
RESULTS

A. Characterisation of fractions from gradient

The construction and operation of the apparatus has been described in detail in the Methodology, as in the method of cell recovery. A calibration chart showing refractive indices of standard solutions of sucrose is shown in Fig. (13). In subsequent experiments, the densities of sucrose solutions in various fractions from the gradient were obtained by puncturing the bottom of the centrifuge tubes and letting the fractions drip out. The density of the various fractions from an illustrative experiment is shown in Fig. (14), which verifies the linearity of the gradient. This data was obtained after centrifuging a sample of cells to ascertain that the passage of cells through it did not upset the gradient by generating possible convection currents. Fig. (15) shows the distribution of cells recovered from a similar gradient after centrifugation. 10 ml gradients were employed in the earlier experiments to facilitate more rapid cell recovery. The nucleated cells were recovered in 3 fractions, which approximate the distribution profile of the bulk of the erythrocytes. The fraction at the top of the gradient contains a proportion of nucleated cells that is heavily contaminated with erythrocytes. The last fraction was very cellular and mostly composed of cells which rapidly sedimented to the bottom. The two peaks of nucleated cells and erythrocytes from the top of the gradient contained cells of different density profiles, being washed down by the travelling meniscus from the sides of the centrifuge tube. The apparatus

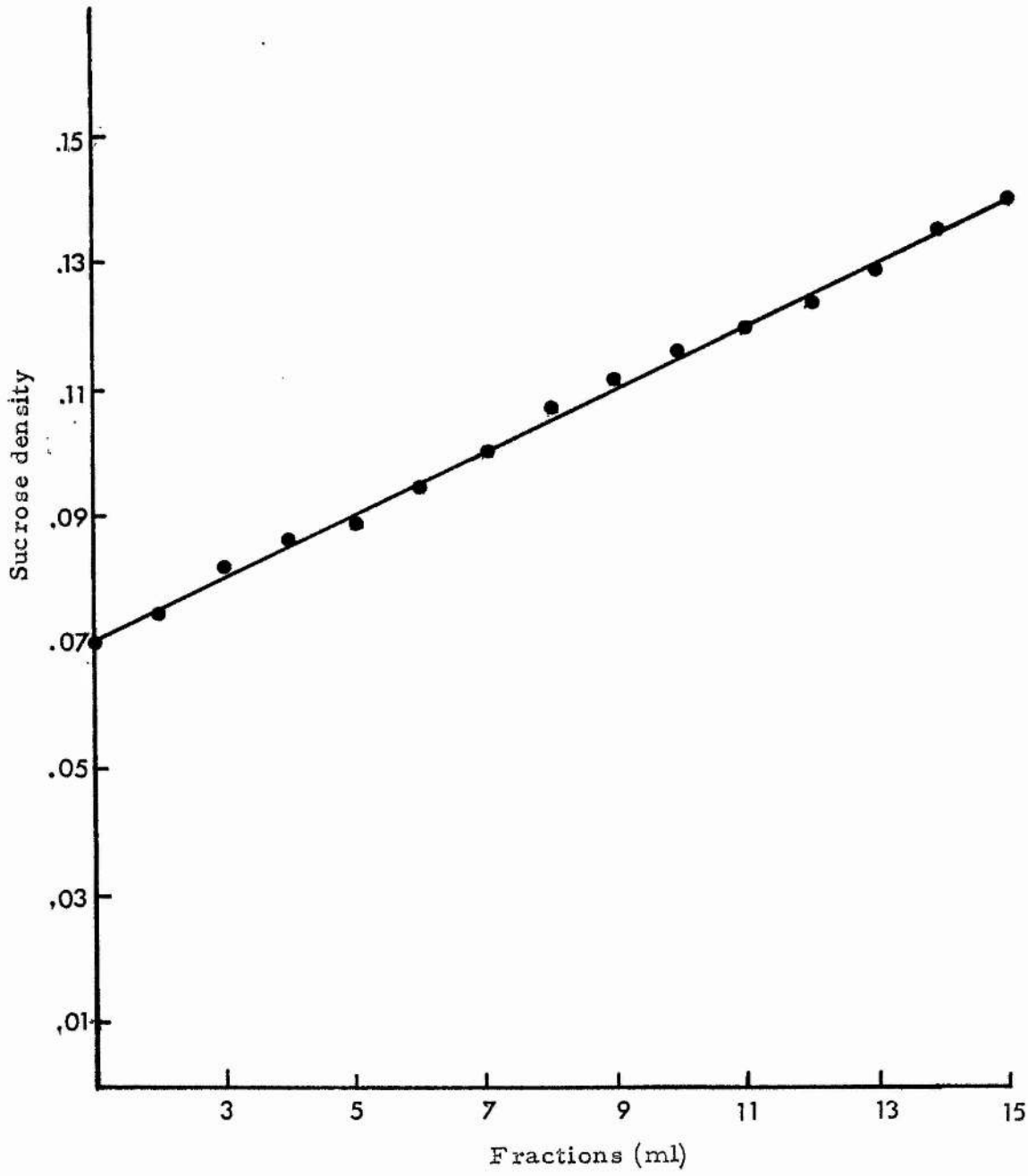
Refractive indices of standard sucrose solutions

Fig. (13)



Sucrose density of fractions from gradient (downward collection)

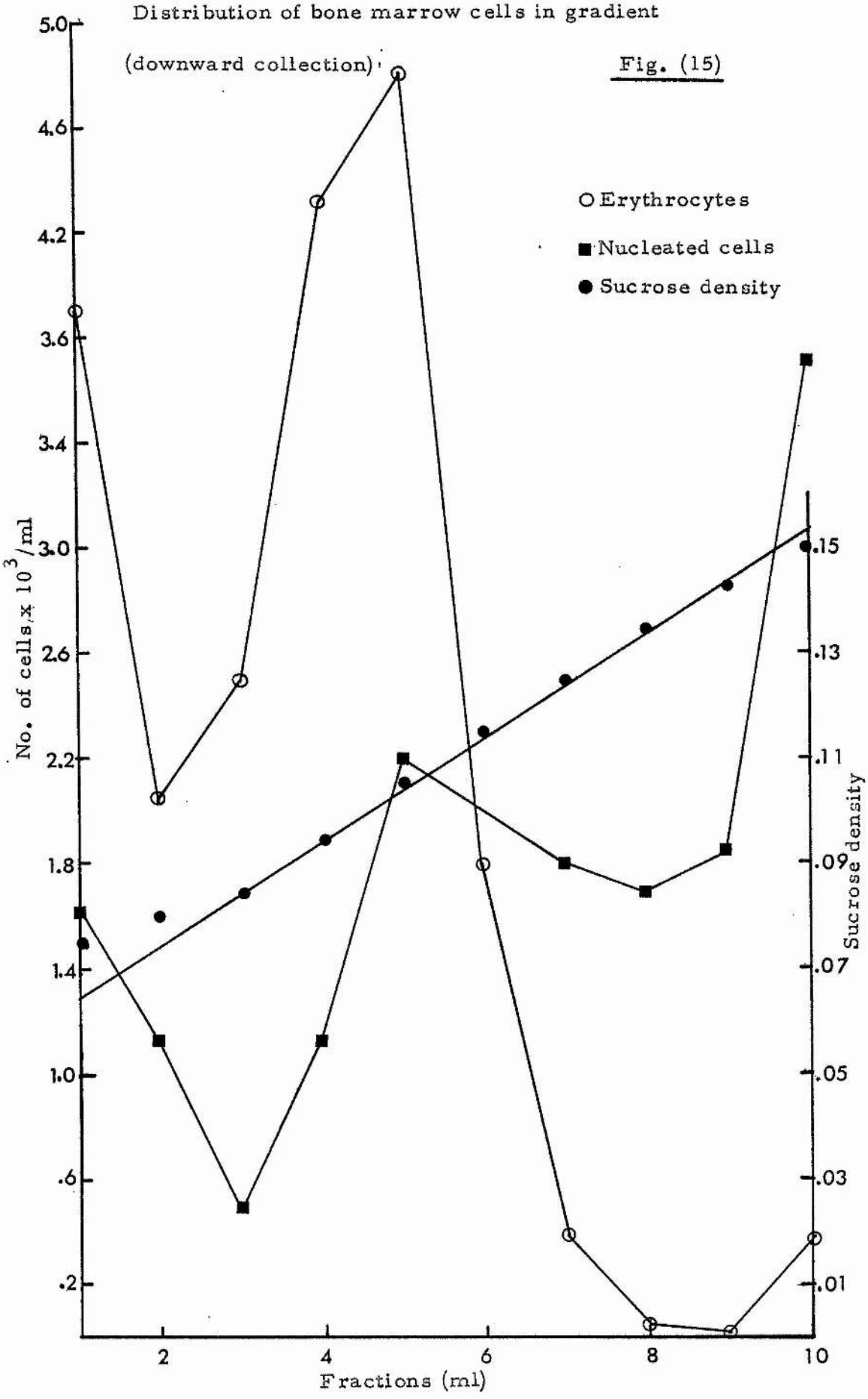
Fig. (14)



Distribution of bone marrow cells in gradient

(downward collection)

Fig. (15)



and method of recovery were modified to overcome this artifact, by recovering the fractions by upward displacement using a denser (20%) sucrose solution.

Fig. (16) shows the density of fractions isolated by upward displacement and the distribution is seen to be similar to the previous procedure. Figs. (17) and (18) show the profile of cells recovered from 10 ml gradients by upward displacement. The shoulder of peaks seen at the top of the gradient previously are lost in the modified method of recovery. A few fractions of nucleated cells exhibit a similar distribution profile to the erythrocytes and are thought to contain the bone marrow lymphocytes. Bone marrow cells from normal animals and those previously treated with HN_2 were separated on the gradients. Plate (70) shows a cytocentrifuge preparation of unfractionated bone marrow cells from HN_2 treated mice. Large undifferentiated blast cells, small/medium lymphocytes and surviving erythrocytes after hypotonic shock are evident. Plate (71) shows those fractions of cells recovered with the erythrocyte-rich layer after centrifugation of HN_2 treated bone marrow cells. Cells resembling small/medium lymphocytes predominate. Plate (72) shows the cells recovered from the penultimate fraction (no. 9), large blast cells and immature granulocytes are the more characteristic cells recovered in this fraction. Plate (73) shows cells from fractionated normal bone marrow recovered in the erythrocyte-rich layers, these fractions were believed to include the bone marrow lymphocytes. In both these experiments, the erythrocytes were found to be located between approx. 0.07 and 0.09 g/ml of sucrose, the peak of nucleated

Plate (70) Cytospin preparation of bone marrow cells from nitrogen mustard (HN_2) treated mice. Large, undifferentiated blast cells and small/medium lymphocytes are seen.

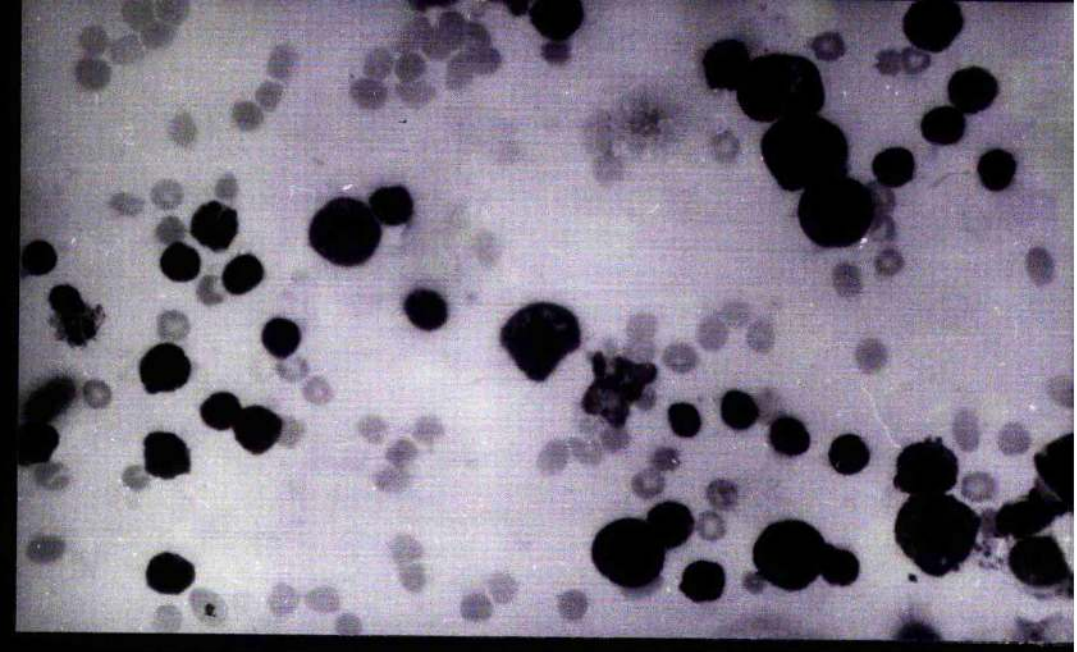
J and G x 480

Plate (71) The proportion of small/medium lymphocytes from above are enriched after separation on sucrose density gradients.

J and G x 480

Plate (72) Large blast cells and immature granulocytes are recovered at the bottom of the gradient.

J and G x 480



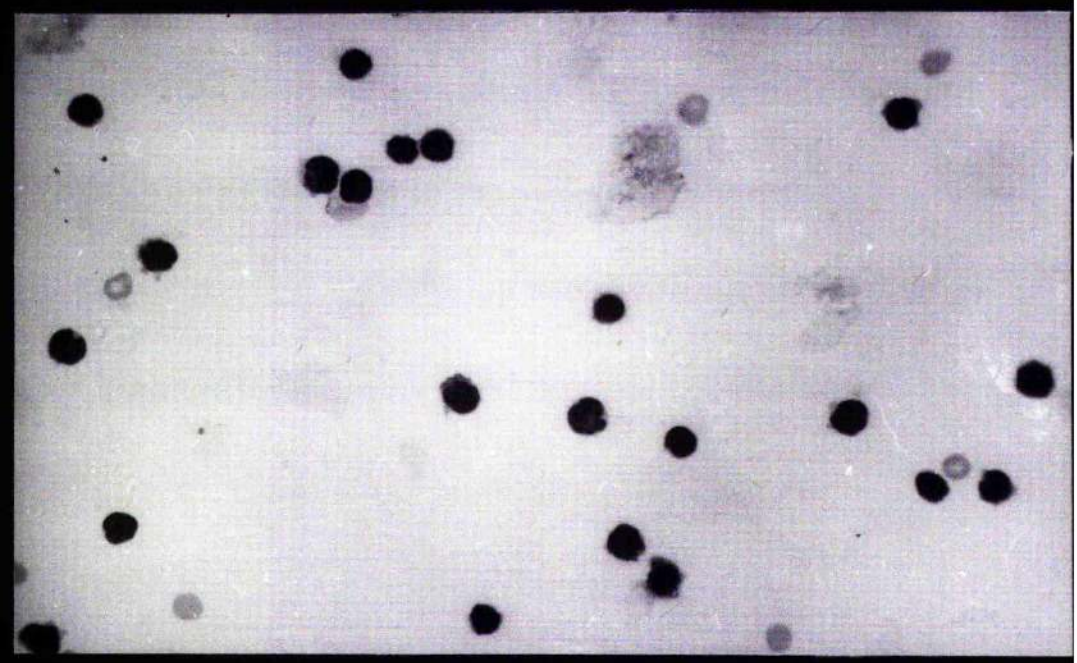
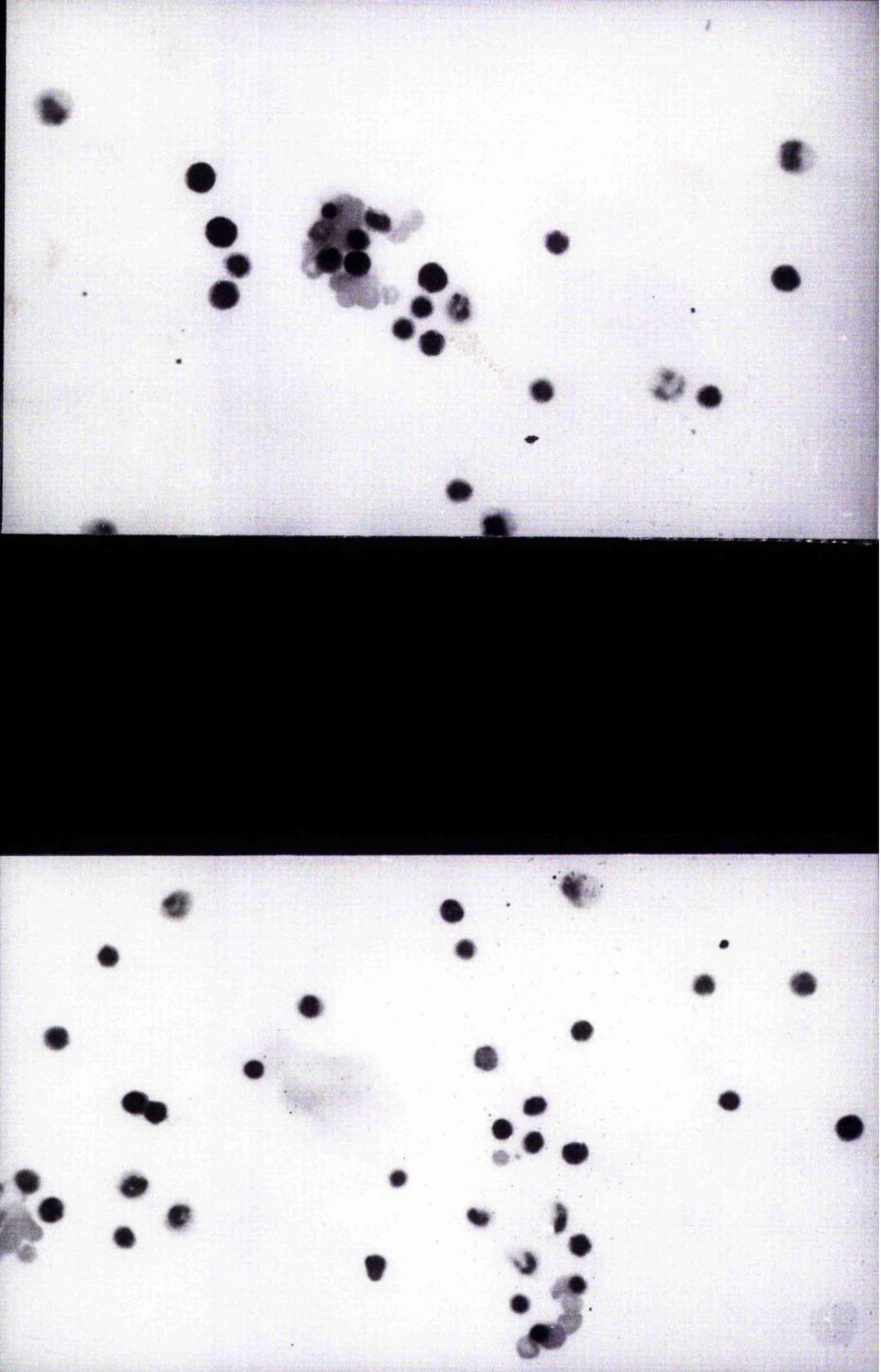


Plate (73) Bone marrow lymphocytes from normal animals that are recovered with erythrocytes after gradient separation.

J and G x 480

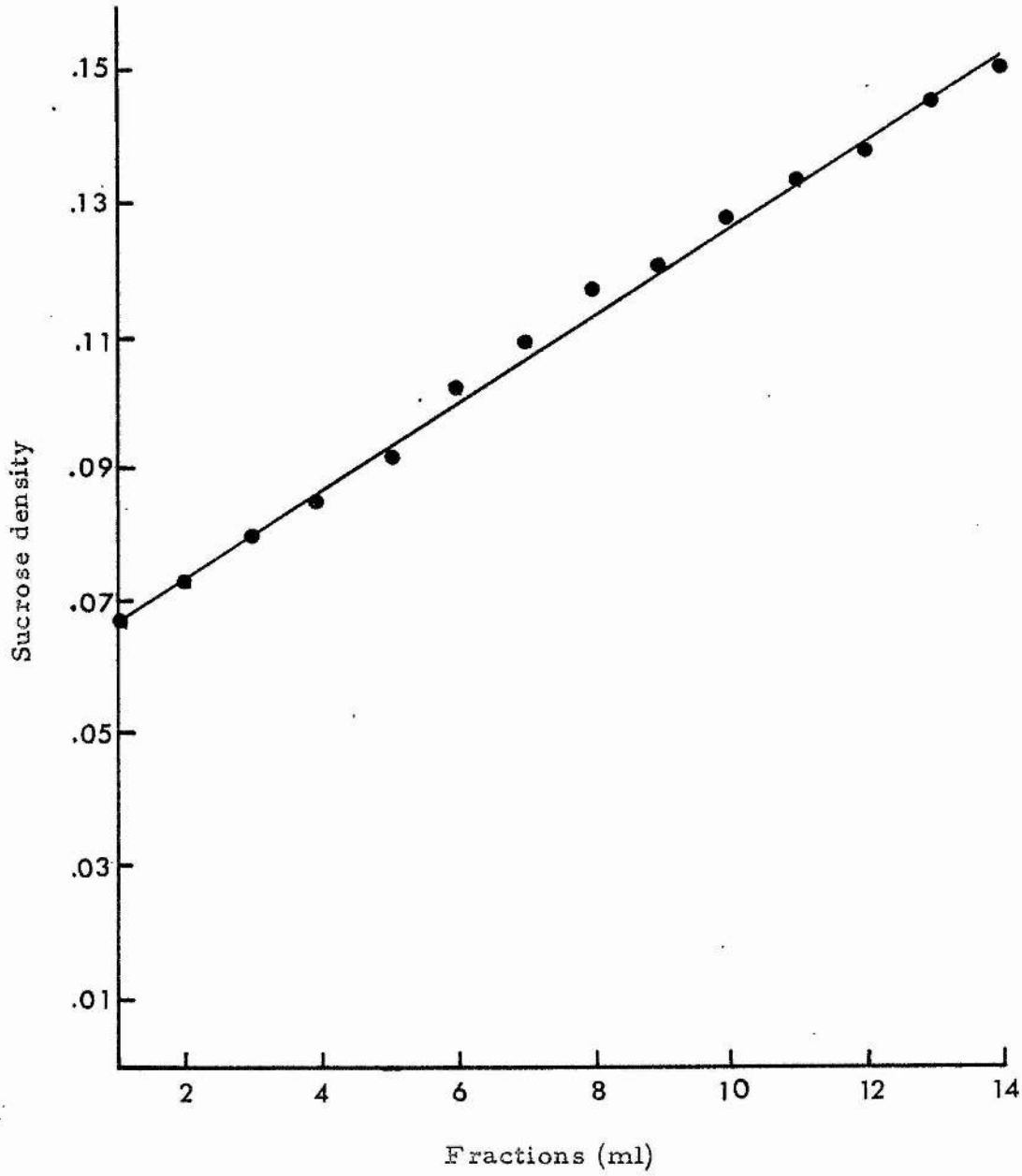
Plate (74) As above, but from a different separation.

J and G x 480



Sucrose density of fractions from gradient (upward collection)

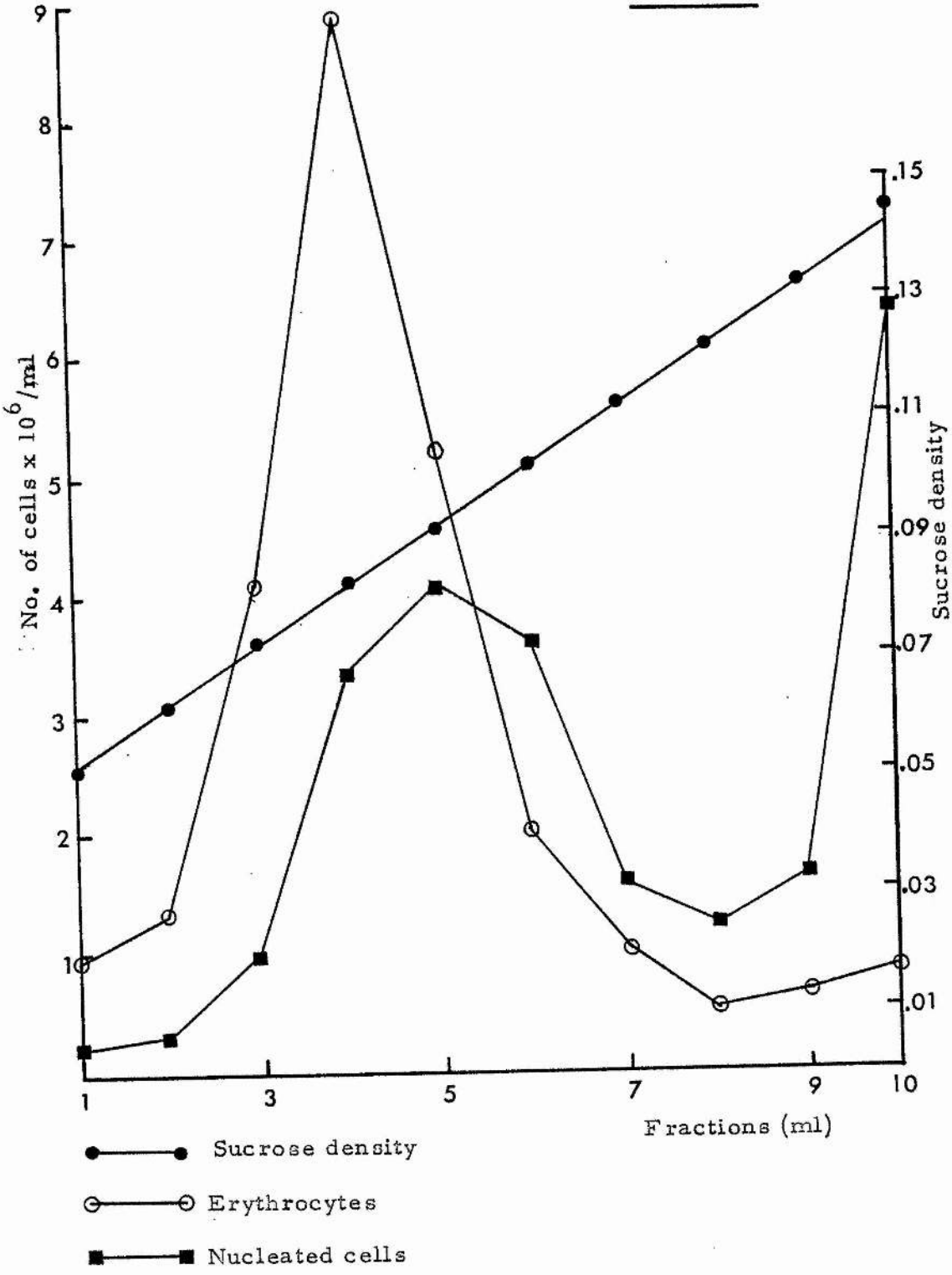
Fig. (16)



Distribution of cells in gradient (upward displacement)

Normal bone marrow

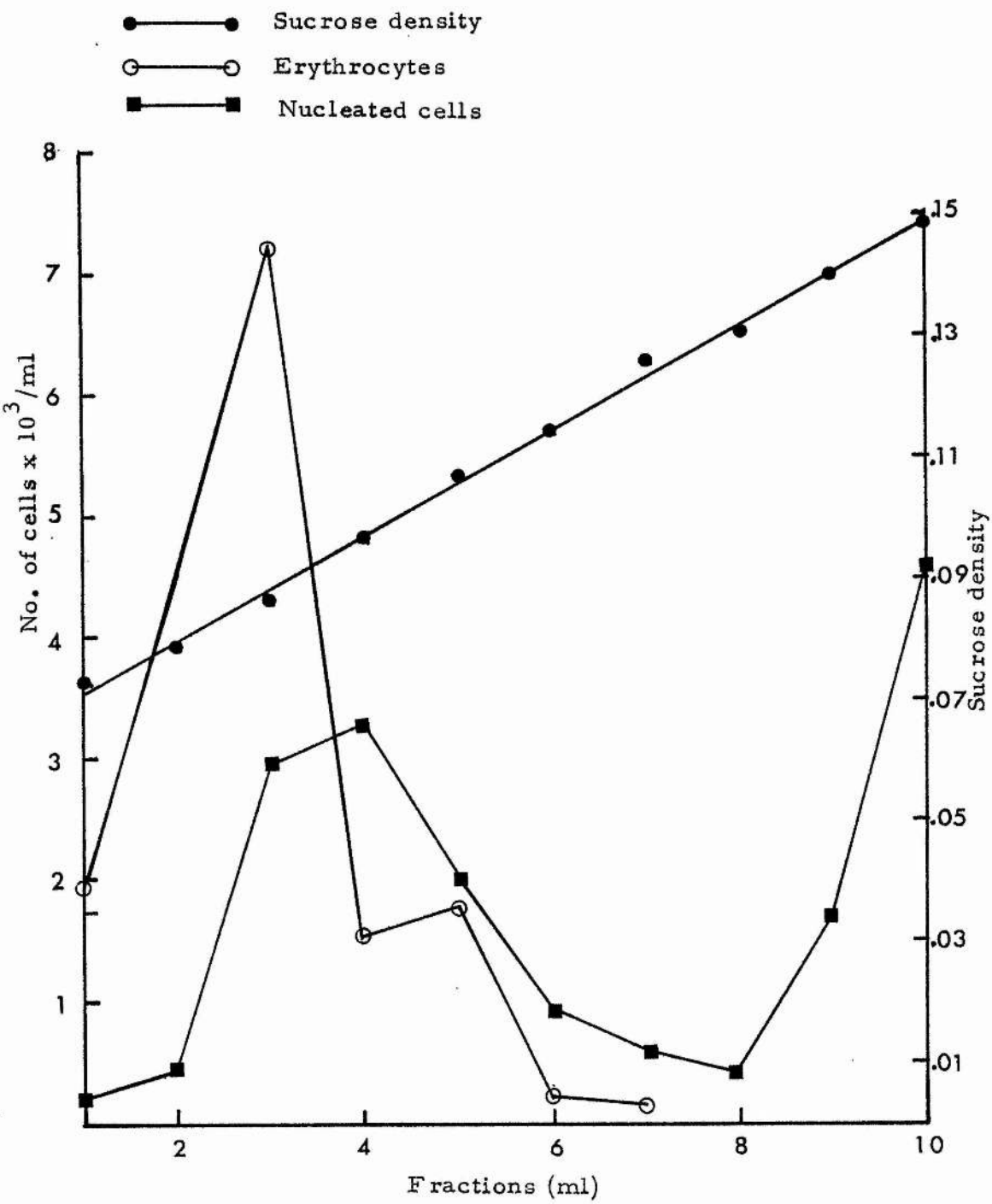
Fig. (17)



Distribution of cells in gradient (upward displacement)

HN_2 treated bone marrow

Fig. (18)



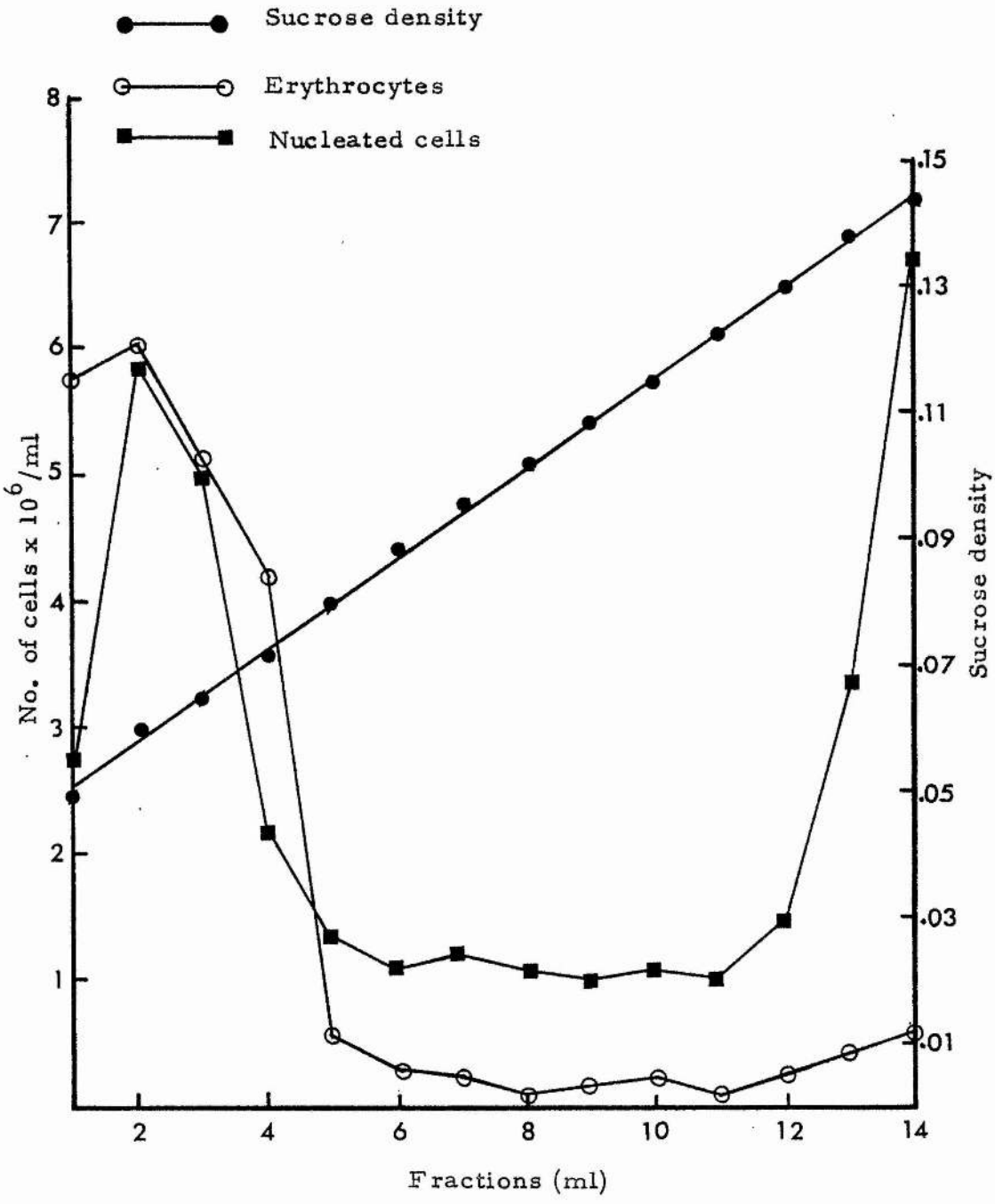
cells preceded these by one fraction. A better separation was achieved by using a larger 14 ml gradient (Fig. 19). The lymphocyte peak closely approximated the erythrocyte recovery profile, a cytocentrifuge preparation from this erythrocyte-rich layer shows a predominance of cells with lymphoid morphology (Plate 74). In this experiment, the erythrocytes were recovered nearer the top of the gradient, corresponding to sucrose densities of 0.05 - 0.07 g/ml. In these cell separation experiments, the numbers of cells/fraction (1 ml) were enumerated using a Coulter counter. Thus any errors are standard throughout the fractions, this being the errors inherent in the instrument.

In a similar experiment, erythrocytes were previously lysed by hypotonic shock and the nucleated cells were centrifuged on a 14 ml gradient. The majority of nucleated cells were recovered between fractions 4-6, corresponding to sucrose densities of 0.08-0.095 g/ml (Fig.20). The cells from this experiment were recovered, pooled into two groups: (A), comprising fractions (1-7) containing the bone marrow lymphocytes (BML) and (B), comprising fractions (8-14) which constituted the bone marrow residue (BMR). Both groups of cells were cultured for 4 days without PHA and their rate of spontaneous DNA synthesis was assayed by the uptake of ^{125}IUdR. Table (13) shows that no significant differences exist between these groups.

Up to a $\frac{1}{4}$ of normal bone marrow cells were found to be erythrocytes; in HN_2 treated marrow, they were the predominant

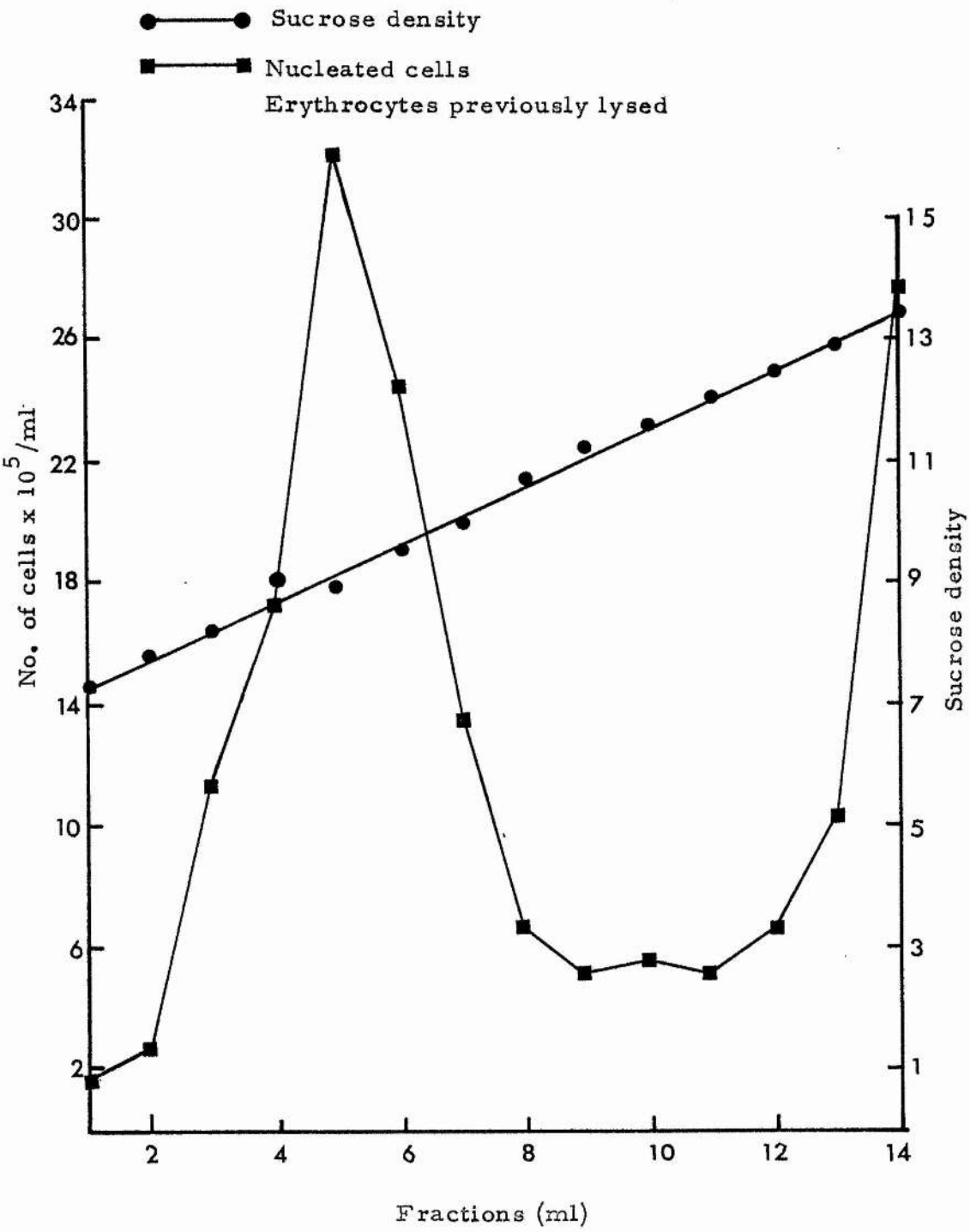
Distribution of cells in gradient (upward displacement)

Fig. (19)



Distribution of cells in gradient (upward displacement)

Fig. (20)



cells where approx. 10^6 nucleated cells were obtained by flushing out the femoral cavity. Approx. a $\frac{1}{3}$ of the nucleated cells in normal bone marrow had lymphoid morphology. This proportion was enriched by gradient centrifugation to between 65-80%. Approx. a $\frac{1}{3}$ of the nucleated cells loaded onto the gradient were recovered in the BML fraction after centrifugation.

B. Effect of sucrose on PHA responsiveness

In these experiments, the cells were exposed to sucrose for varying lengths of time; therefore the effect of prior incubation with 10% sucrose for 30 minutes, on the subsequent PHA response of whole, unfractionated bone marrow cells was investigated. The cells were incubated for 4 days with 20, 10 and 5 μg/ml of PHA. Table (14) indicates that at most cell concs. and doses of PHA, there were no significant differences between the groups. The differences, when present, showed that sucrose treated cells were stimulated to a slightly greater extent.

C. PHA responses of fractionated cells

The cells recovered from the gradient were pooled into two groups as before, bone marrow lymphocytes (BML) and residue (BMR). The BML comprised the nucleated cells in the erythrocyte-rich fractions plus 2 x 1 ml fractions below this layer; the BMR comprised the cells from all fractions below the former, in the denser layers. They were cultured with PHA at a high dose of 20 μg/ml for a total period of 4 days at a cell conc. of 7×10^5/well.

Table (13). Spontaneous DNA synthesis of bone marrow cells isolated from gradient

Cells	Mean cpm ± SD	SE	Sample (N)
BML	111 ± 39	9	19
BMR	83 ± 18	5	14

Cells cultured at 10^6/well

No difference between above groups ($p > 0.05$)

Table (14). Effect of prior exposure to sucrose on PHA response of bone marrow cells

Sample (N) = 5

PHA Dose µg/ml	Cell conc. x 10^5	Mean cpm ± SD	SI ± SD	Treated cells Mean cpm ± SD	Treated cells SI ± SD	p
20	10	369 ± 120	1.7 ± 0.3	440 ± 110	3.9 ± 2.5	< 0.02
	5	281 ± 117	1.2 ± 0.5	544 ± 57	5.0 ± 1.3	< 0.001
	2	378 ± 284	1.9 ± 1.5	405 ± 111	2.3 ± 0.7	> 0.1
10	10	256 ± 43	1.2 ± 0.3	330 ± 86	2.9 ± 1.9	< 0.02
	5	363 ± 94	1.6 ± 0.6	351 ± 26	3.2 ± 0.8	< 0.001
	2	166 ± 56	0.9 ± 0.3	327 ± 21	1.8 ± 0.6	< 0.001
5	10	297 ± 41	1.4 ± 0.3	192 ± 11	1.7 ± 1.0	> 0.1
	5	328 ± 42	1.5 ± 0.2	201 ± 40	1.9 ± 0.6	> 0.05
	2	243 ± 46	1.3 ± 0.3	240 ± 28	1.3 ± 0.4	
-	10	214 ± 44		114 ± 70		
	5	226 ± 12		108 ± 27		
	2	194 ± 34		179 ± 57		

Table (15). PHA response of bone marrow cells separated on gradients

Cells ± PHA	Mean cpm ± SD	SI ± SD	Sample (N)	p
BML + PHA	347 ± 34	1.9 ± 0.3	6	< 0.001
BML only	175 ± 29		6	
BMR + PHA	46 ± 11	1.1 ± 0.6	4	> 0.1
BMR only	42 ± 20		3	

Difference between BML and BMR ($p < 0.01$)

Cells at 7×10^5/well. PHA at 20µg/ml. Cultured for 4 days.

Table (15) shows their proliferative response assayed 24 h after the addition of 0.2μCi of ^{125}IUdR. The response obtained is meagre, with a stimulation index of 1.9 for the BML whereas the BMR did not respond to PHA. In a separate experiment, the effect of autologous mouse serum supplement on the PHA response of BML was investigated, and was not found to be significantly different from that of foetal calf serum (Table 16).

The BML and BMR fractions were cultured for a longer period of 5 days at various cell concs. and PHA at 20μg/ml. There are moderate differences between the groups which are more apparent at the higher cell concs. and is insignificant at the lowest. Lymph node cells were cultured as a contrast, at a conc. of 2 x 10^5/well with PHA at 20 and 4μg/ml. It can be seen that at 20μg/ml, PHA inhibits the proliferative response of lymph node cells. BMR was stimulated to a lesser extent than the BML and have slightly lower stimulation indices where there is a significant difference (Table 17). In a subsequent experiment, BML and BMR were cultured for 4 and 5 days at a cell conc. of 7 x 10^5/well with PHA at varying doses of 16, 8, 4 and 2μg/ml. Table (18) shows that at these lower doses of PHA, the proliferative response is negligible in the BML and is absent in nearly all the BMR groups. A longer culture period of 5 days did not elicit a greater response, except at 8μg/ml of PHA, where a slightly increased stimulation was observed.

Table (16). Effect of mouse serum supplement on PHA response

Cells ± PHA	Serum supplement	Mean cpm ± SD	SI ± SD
BML + PHA	Mouse serum	219 ± 70	1.5 ± 0.6
BML only	Mouse serum	144 ± 38	
BML + PHA	FCS	354 ± 193	1.8 ± 1.0
BML only	FCS	193 ± 51	

Sample (N) = 6

No significant difference between serum effects ($p > 0.1$)

Table (17). PHA response of bone marrow cells separated on gradients

Cells ± PHA	Cell conc. x 10^5/well	Mean cpm ± SD	SI ± SD	Sample (N)	p
BML + PHA	8	410 ± 50	2.3 ± 0.4	3	< 0.01
	4	278 ± 83	2.1 ± 0.7	5	< 0.01
	2	175 ± 33	1.5 ± 0.4	5	< 0.01
BML only	8	175 ± 24		3	
	4	132 ± 25		6	
	2	114 ± 20		5	
BMR + PHA	8	357 ± 26	1.4 ± 0.4	6	< 0.01
	4	291 ± 43	1.2 ± 0.3	6	> 0.05
	2	297 ± 25	1.8 ± 0.6	5	< 0.001
BMR only	8	257 ± 67		6	
	4	239 ± 38		6	
	2	163 ± 51		6	
Controls					
lymph node cells + PHA 20 µg/ml	2 x 10^5	231 ± 66	3.1 ± 1.1	6	
lymph node cells + PHA 4 µg/ml	2 x 10^5	14802 ± 1149	202 ± 44.4	12	
lymph node cells only	2 x 10^5	73 ± 15		5	

Cells cultured for 5 days

Differences between BML and BMR only at higher cell concs. ($p < 0.001$)

Table (18). PHA response of gradient enriched bone marrow cells: Dose study and comparison of culture periods

Cells	PHA Dose µg/ml	4 day culture			5 day culture		
		Mean cpm ± SD	SI ± SD	p	Mean cpm ± SD	SI ± SD	p
BML	16	231 ± 57	2.1 ± 1.0	0.01	206 ± 68	3.0 ± 1.2	< 0.01
	8	205 ± 48	1.9 ± 0.9	0.02	224 ± 43	3.3 ± 0.9	< 0.001
	4	164 ± 40	1.5 ± 0.7	0.05	139 ± 30	2.1 ± 0.6	< 0.01
	2	176 ± 27	1.6 ± 0.3	0.05	80 ± 18	1.2 ± 0.4	> 0.1
	-	110 ± 46			68 ± 13		
BMR	16	103 ± 19	0.9 ± 0.3	0.1	158 ± 30	2.4 ± 1.3	< 0.01
	8	89 ± 23	0.8 ± 0.3	0.1	132 ± 33	2.1 ± 1.2	< 0.02
	4	91 ± 34	0.8 ± 0.3	0.1	63 ± 27	0.9 ± 0.6	> 0.1
	2	77 ± 28	0.7 ± 0.3	0.005	105 ± 26	1.6 ± 0.9	> 0.05
	-	108 ± 23			64 ± 32		

Sample (N) = 5

DISCUSSION

The generation of a linear gradient of sucrose by the apparatus constructed was considered reproducible. For a number of reasons, the gradient was not considered to separate cells by density equilibration, because of the distribution profile of the recovered cells. When centrifuged for prolonged periods, all the cells were recovered in a pellet. As cells with a high nucleus: cytoplasm ratio are considered to be the denser cells, small lymphocytes would be expected to be recovered at the lower end of the gradient, in the denser fractions; in these separations, small lymphocytes were recovered nearer the top of the gradient. Furthermore, in replicate experiments, the erythrocyte peak was found to correspond to slightly different densities of sucrose, probably as a consequence of fluctuations in centrifugation speed and temperature. It is considered that the cells in these separation procedures were fractionated on the basis of size rather than density. It can be seen from the cytocentrifuge preparations that immature granulocytes and larger cells were recovered from the denser fractions. However, as the bone marrow lymphocytes and erythrocytes were closely associated, the latter provided an easily identifiable marker; these cells were thought to correspond to the cells constituting the lymphocyte-rich fractions isolated after density centrifugation by Osmond and Yoshida (1971) and Press and Rosse (1977a). The cells isolated by this method range in size from 7-11 microns in diameter and correspond to small and medium lymphocytes. It is not known whether these cells also included the bone marrow transitional cells

described by Yoffey (1966) and reviewed by Rosse (1976), from which small lymphocytes are thought to arise. Their size range of 9-12 microns would not preclude them from this fraction. Transitional cells, unlike small lymphocytes are thought to synthesise DNA and consequently incorporate ^{3}H TdR without mitogenic stimulation. When pooled BML and BMR fractions were incubated with ^{125}IUdR, higher levels of incorporation were not detected compared to whole unfractionated marrow, and no noticeable differences between the BML and BMR fractions were seen. As the BMR comprised a heterogenous mixture of cells, it is difficult to attribute any characteristic to any one particular type of cell or to compare their PHA responses with the the lymphocyte enriched fraction.

The PHA responsiveness of both BML and BMR fractions were minimal with maximum stimulation indices of 3.3 and 1.8 respectively. These values are vastly different from that of Press and Rosse (1977a) who report stimulation indices of between 5-40. The markedly divergent results obtained in this study from their's can be attributed to a few reasons, assuming that similar cell populations were isolated in both investigations. In both studies, the nucleated cells distributed with the erythrocytes were recovered and subsequently cultured. According to these investigators, the sensitivity of their experimental system to detect the PHA reactivity of bone marrow cells depended upon longer incubation periods with doses of PHA very much greater than normally used to stimulate peripheral lymphocytes. They found an ill-defined peak of stimulation between 4-40μg/ml of PHA which was maximal between 4-7 days. In this, study, no marked differences between either day in the PHA response

on days 4 and 5 of incubation were detected; slightly greater stimulation indices for the BML were obtained on day 5. The culture conditions were not considered to adversely affect lymphoid growth as control lymph node cells responded very well at 4μg/ml of PHA, but 20μg/ml was found to be definitely inhibitory. In a dose study, fractionated BML and BMR did not elicit a marked stimulation on either day or at any of the lower doses of PHA or cell concentrations. Press and Rosse (1977a) incubated the cells at higher concentrations of 10^6/ well, whereas fewer cells were used in this study as cells recovered from the gradient were always at a premium. They report that the blastogenic response of BML levelled off at 2 x 10^6 cells/well. In a preliminary experiment, cells at this conc. were cultured, but were found not to survive after 3 days in culture; this very high conc. of cells was considered not favourable for culture. These authors claim that the stimulation of lymph node cells reached a plateau at 10^6/well. In this study, lymph node cells were always cultured at 2 x 10^5/well which was found to be the optimum conc. They also utilised 3H TdR to measure DNA synthesis, whereas in this study, ^{125}IUdR was used to assess cell proliferation. Differences in the level of incorporation of different isotopes should not affect the comparison of stimulation indices from different studies. These investigators report that 1% autologous mouse serum supplement was found to be optimal for stimulation; in contrast, this was found to be indifferent in this study. The differential effects caused by various batches and sources of serum supplements are a consideration to be taken into account. It is perhaps noteworthy that certain differentiation antigens have been spuriously expressed on cells of other differentiation lineages.

The Thy 1 antigen has been detected on Wehi/3 cells, a monocytic cell line in the presence of certain batches of serum and not others (E.G. Wright, personal communication).

The active moieties in mitogens are thought to be glycoproteins which bind to sugar residues on cell surface membranes (Lis and Sharon, 1973). The possibility that prior exposure to sucrose might competitively inhibit the mitogenic effect of PHA by carry over of cell-bound sucrose was discounted as no consistent differences were seen in the responses of the groups of cells. The level of stimulation was uniformly low at all doses of PHA and concentrations of cells tested. However, the design of the experiment necessitated the use of unfractionated bone marrow cells.

Press and Rosse (1977a) postulate that the BML cells that respond to PHA are precursors of T cells, which are first induced to functional maturity by PHA before a blastogenic response occurs, thus accounting for the longer incubation periods needed. They compare their acquired PHA reactivity with the induction of surface markers by thymosin and non-thymic agents on bone marrow cells reported in other studies (Woody et al, 1973 and Scheid et al, 1973). Such inducible cells in the bone marrow must include post-thymic precursors of T cells. It is not known whether post-thymic precursors are also found in the lymphomyeloid tissues of the marrow parenchyma in addition to those in the vascular bed. If not, their numbers would be expected to be fairly small, and prolonged incubation periods would be expected, first to amplify the PHA responsive population, and subsequently to detect a blastogenic response. Such an explan ation would account for the kinetics of the PHA response, which shows an extended plateau

between 4-7 days as reported by these investigators. However, they refute this possibility by obtaining similar PHA responsivity using bone marrow cells from nude mice (Press and Rosse 1977b).

In this study, the BML fraction was tested for the Thy 1 antigen and found on approx. 4% of the cells (Chapter 1). Furthermore, these cells when injected into an embryonic thymus, depleted of its own lymphoid cells, were not able to repopulate the organ in vitro as far as could be ascertained (Chapter 2). This latter finding can be interpreted to suggest that the BML fraction does not contain pre-thymic precursors able to repopulate the thymus, under the conditions of the experimental system. Thus, if the proposed precursor T cells are in fact post-thymic, a greater proportion of the BML might be expected to be Thy 1 positive, in contrast to the 4% detected in this study. However, there is evidence that the prevalence of Thy 1 positive cells in the bone marrow of mice differs between strains, and is thought to be absent in CBA mice (Raff, 1971). Furthermore, Blomgren and Svedmyr (1971) found that α-Thy 1 + C' treatment abolished the PHA reactivity of peripheral lymphocytes but had no effect on the response of bone marrow cells. Thus any connection between PHA reactivity and expression of Thy 1 have to be interpreted with caution, considering the anomalous properties of bone marrow lymphocytes.

CHAPTER 4

Cytokinetics of the thymus during pregnancy

INTRODUCTION

The first major study of lymphopoiesis in the thymus was undertaken by Metcalf and co-workers and their findings are reviewed by Metcalf (1964). The murine thymus is relatively large and constitutes 15-35% of the total lymphoid mass of the animal; the proportion being greatest in the neonate and less in the adult following post-pubertal involution, with increasing age. It is recognised that cell proliferation in the thymus is far greater than in any other lymphoid organ. However, most of this lymphopoiesis is apparently futile as it is parallelled by a high incidence of cell death in the organ, most of the newly formed lymphocytes having a very short life-span. Lymphocyte proliferation in the peripheral lymphoid organs is a consequence of antigenic challenge; in the thymus, it is not provoked by 'foreign' stimuli as it is not significantly different in germ-free animals than to those exposed to a normal environment. Several factors contribute to the homeostasis of thymic lymphopoiesis; hormonal influences mediated by both gonadal and adrenocortical steroids have a marked effect on the cellularity of the thymus, leading to a severe lymphopenia (Castro, 1974 and Dougherty et al, 1964). A factor contributing to the age-related atrophy of the organ is the increased levels of steroid hormones after puberty. Yet the thymus appears to exhibit some degree of independence to influences by systemic factors, neonatal thymuses transplanted to older animals exhibited the growth characteristics typical of the graft's own age and strain (Metcalf, 1961).

The thymus has been implicated in the susceptibility to leukaemogenesis, its incidence being reduced after thymectomy

(Kaplan, 1950 and McEndy et al, 1944). Possible regulatory cells responsible for determining these effects are likely to be the reticuloepithelium of the thymus. It has been claimed that in vitro monolayers of non-lymphoid cells derived from thymuses of AKR mice, a strain with a high incidence of spontaneous leukaemias, are able to transform thymocytes upon co-culture (Waksal et al, 1976). In vivo, a greater proportion of mitotic cells were seen in close association with PAS positive, presumably epithelial cells by Metcalf and Ishidate (1961), who postulated that an interaction with these cells or their secretory products initiated mitosis. The cortical thymocytes are probably pre-determined to undergo a finite number of mitoses before they migrate to the medulla by which time they have expended their proliferative potential.

The thymus is known to involute rapidly following x-irradiation and is subsequently repopulated by blood-borne stem cells (Ford et al, 1966). Fabrikant and Foster (1971) have suggested that the predominant factor controlling thymic lymphopoiesis is the availability of new stem cells. The cellularity of the thymus is also perturbed during pregnancy, causing a significant aplasia which continues during and recovers after lactation (Gregoire, 1947).

The increased steroid hormone levels during pregnancy are thought to be at least incidentally partly responsible for the transient involution of the thymus. However, the final effect would be seen in the immunological responsiveness of the animal, which is of consequence to the survival of the foetus as a homograft. The immune status of the animal during pregnancy has been investigated

by a number of investigators and found to be perturbed. Blocking antibodies (Hellstrom et al, 1969) and suppressor mechanisms (Murgita et al, 1977 and Olding and Oldstone, 1974) have been implicated in maintaining what is essentially a parabiotic union of disparate antigenicity. However, there is evidence that the pregnant animal may itself evoke an immune response to foetal antigens of paternal genotype (Rocklin et al, 1973 and Maroni and Parrott, 1973). In addition to histoincompatible antigens, there are differences between foetal and maternal tissues, the embryonic antigens,which might be immunogenic even in syngeneic matings. Tyndall et al (1972) have observed similarities between embryogenesis and oncogenesis, suggesting that factors affecting immune functions in the mother are comparable to those of tumour-bearing animals, and might be caused by similar mechanisms. It is relevant to these considerations that kinetic studies of the thymus have been carried out after in vivo treatment with prednisolone, which might mimic the endocrinological milieu during pregnancy (Zaitoun et al, 1979) and in tumour-bearing animals (Aherne et al, 1980).

During pregnancy, the spleen also undergoes a change in its cellular profile, splenomegaly has been observed in pregnant mice by Fowler and Nash (1968) and by Maroni and De Sousa (1973). This could be caused either by lymphocytic proliferation during an allergic response to foetal antigenic stimuli, or may be a reflection of increased haemopoietic activity during pregnancy. The murine spleen is considered to function as a site of extramedullary haemopoiesis in conditions of stress, when it is capable of greatly increasing its erythropoietic activity (Turner et al, 1967).

PROCEDURE

In this study, the gross changes in the thymus and spleen at different stages of gestation were observed in an initial investigation. The cellularity of the thymus was also determined at these intervals. These times were chosen to represent early (5 days), mid (10 days), and late (15 days) stages of gestation, and at parturition. As parturition generally occurred between 19-21 days, subsequent experimental protocols were carried out at the late gestational stage of 15 days which was considered to be more consistent. Syngeneic matings between CBA mice were used unless otherwise stated.

A more detailed study of the cell proliferation characteristics in normal (non-pregnant) and in pregnant animals was undertaken. The animals were treated with vincristine sulphate, a stathmokinetic agent in vivo and the thymuses removed after varying times, fixed and processed for histology. Cell proliferation in the thymus was assessed by enumerating the number of cells arrested in metaphase seen at a X8000 magnification. The number of dividing cells were counted either/field of view or within the area defined by a grid incorporated into the eye-piece of the microscope, in various regions of the thymus. At this magnification, the grid covered an area of 0.004 sq mm of the section. Each thymic lobe was considered as one sample, from which sections were made and replicate counts were taken from each slide. The cellularity in each region of the thymus was calculated by similarly counting the total number of nuclei, both in metaphase and interphase. As

such readings would include fragments of nuclei cut at different diameters by the microtome, an overestimation of the total number of nuclei/area would result. Therefore the mitotic index obtained, calculated from the number of metaphase nuclei/1000 lymphoid cells was corrected by the method of Abercrombie (1946). The chord lengths of random metaphase and interphase nuclei were determined by measurement. From the mean chord length, the true nuclear diameters in metaphase and interphase were calculated, and thus the true ratio of metaphase nuclei/total no of dividing cells, by the following correction:-

$$Nc = Ne \left(\frac{T}{T+L} \right)$$

where Nc = corrected nuclear diameter

Ne = experimentally determined nuclear diameter

T = section thickness

L = mean random chord length

Similarly $$Mc = Me \left(\frac{T}{T+L} \right)$$

where the corresponding diameters are of metaphase nuclei.

These correction factors were applied to the number of metaphase and interphase nuclei in the different regions of the thymus from the subcapsular to the cortex/medulla junction. The cell production rate was determined by calculating the ratio of mitoses/1000 cells in each of the regions of the thymus, and plotting the data against time. A statistical treatment of the data to obtain the linear regression by the method of least squares on an Apple II computer, enabled the cell production rate to be determined from the gradient of the regression lines.

The immune reactivity of pregnant and normal animals was assessed by investigating the PHA responsiveness of their spleen and lymph node cells at a dose of 4μg/ml of PHA. The results represent data from one animal per experimental group. The differential counts of spleen cells correspond to each animal from which the mitogen stimulation data was obtained.

CBA mice were treated with 1μCi of ^{59}Fe to assess the erythropoietic activity in the spleen and bone marrow during pregnancy after syngeneic matings, to further elucidate the causes of the observed splenomegaly.

RESULTS

A. Cellularity of thymus and spleen during pregnancy

The changes in weight and cellularity of the thymus parallel each other as would be expected. After 5 days of gestation, there is a noticeable decrease in these parameters, the trend is seen to continue for the duration of the period of study ie. till parturition. In contrast, there is a pronounced splenomegaly which is most apparent at mid-gestation and decreases thereafter, although it still persists at the termination of pregnancy. In syngeneic matings, the thymus is approx. half its normal size at parturition, whereas the spleen is almost double its normal size at mid-gestation (Table 19 and Fig. 21).

In a supplementary study, the weight and cellularity of the thymus and spleen from CBA mice pregnant by allogeneic matings with C57 males were determined at 15 days of gestation (Table 19). The weight and cellularity of the thymus was not found to be significantly less compared to syngeneic matings; however the degree of splenomegaly was found to be greater at 15 days than it is at 10 days after syngeneic matings when the spleen is at its largest.

B. Cytokinetics of the thymus

In a preliminary study, non-pregnant mice were treated with 1-5 mg of vincristine/kg body wt for a duration of 2 h and the no. of metaphase nuclei in the subcapsular cortex were counted. Although vincristine was effective at all doses tested, a slight peak at 2 mg/kg was detected (Table 20 and Fig. 22). In a further experiment, the frequency of proliferating cells in three regions

Table (19). Change in weight of thymus and spleen during pregnancy

Gestational age (days)	Weight of thymus (mg) ± SD	SE	Weight of spleen (mg) ± SD	SE	Cellularity of thymus cells x 10^6/mg	SE	(N)	p
Control non-pregnant	49.3 ± 8.4	1.5	71.0 ± 9.3	1.6	2.3 ± 0.5	0.08	32	
5	47.4 ± 7.7	2.4	69.7 ± 9.5	3.0	2.4 ± 0.4	0.12	10	
10	34.2 ± 7.2	2.6	120.3 ± 11.5	3.6	2.1 ± 0.5	0.1	12	<0.001
15	24.9 ± 8.2	2.6	110.9 ± 14.3	4.5	1.8 ± 0.3	0.1	10	<0.001
Term	12.8 ± 3.1	1.1	91.8 ± 16.1	5.7	1.1 ± 0.4	0.1	8	<0.001
15 days*	21.8 ± 5.0	1.6	138.5 ± 21.1	6.7	1.3 ± 0.4	0.1	10	<0.01

* In allogeneic matings CBA x C57 , spleen larger than in syngeneic pregnancies ($p < 0.01$) thymus weight not difference ($p > 0.1$).

Other p values are for both thymus and spleen weights compared during syngeneic pregnancies

Changes in size of thymus and spleen during pregnancy

Fig. (21)

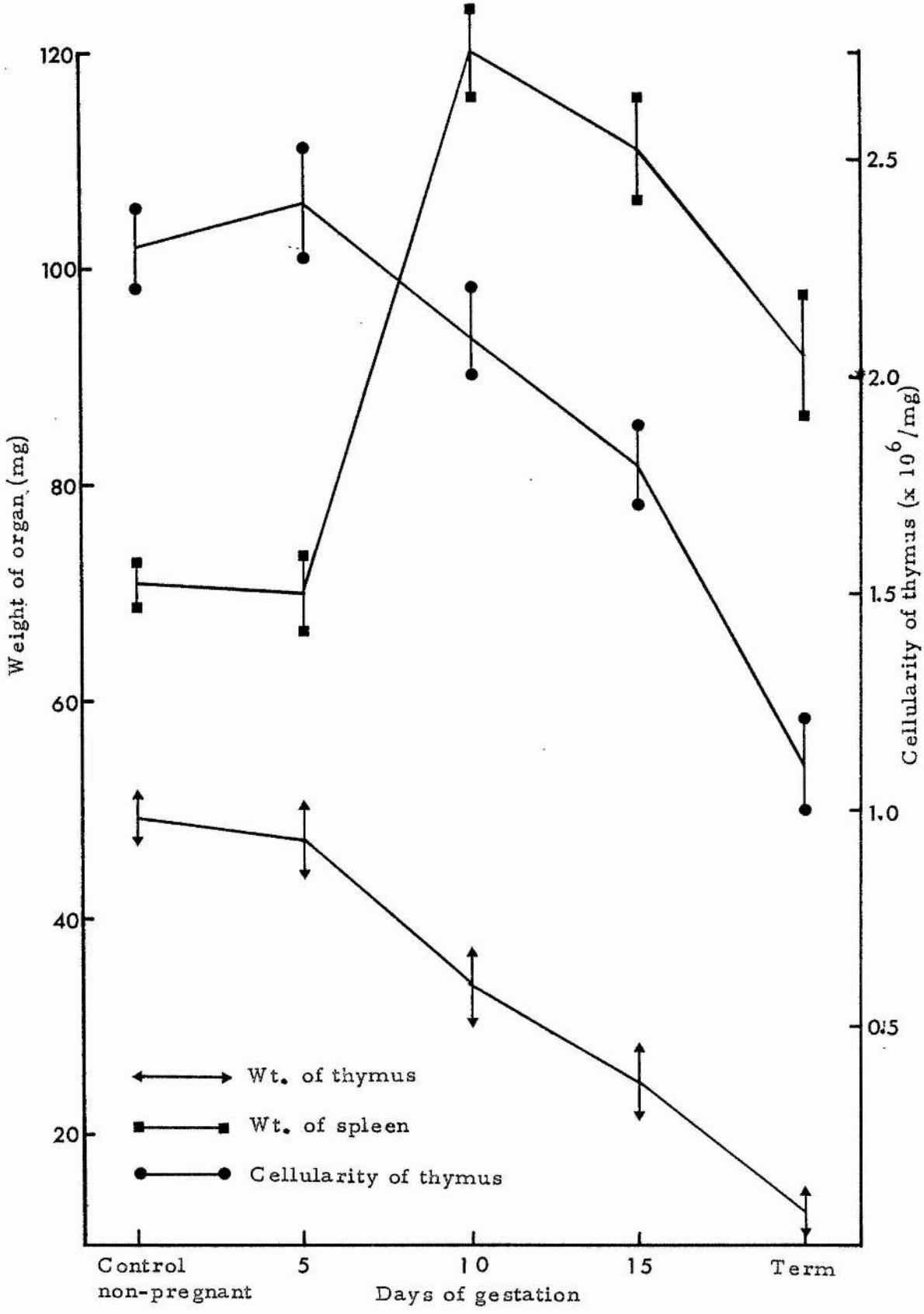


Table (20). Preliminary dose study with vincristine

Dose of vincristine mg/kg body wt.	Mean no. of metaphases in subscapular cortex ± SD	SE
1	18.5 ± 3.5	1.1
2	27.4 ± 8.2	2.6
3	19.0 ± 7.2	2.3
4	20.5 ± 2.5	0.8
5	14.8 ± 3.7	1.2

Sample (N) = 10

Optimal dose = 2 mg/kg ($p < 0.05$)

Table (21). Distribution of mitotic cells in thymic cortex

Region of thymic cortex	Mean no. of metaphases ± SD	SE
Subscapular	36.8 ± 9.4	3.0
Mid-cortex	26.7 ± 9.6	3.0
Cortex/medulla	16.4 ± 12.7	4.0

Sample (N) = 10

Difference between subscapular and cortex/medulla regions ($p < 0.001$)

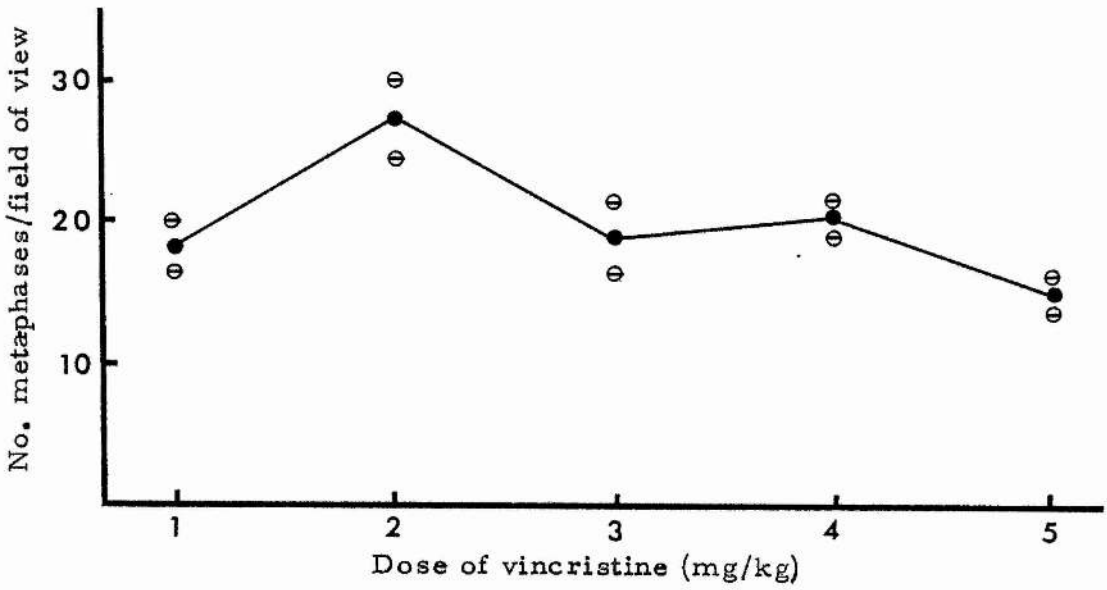
Table (22). Vincristine dose study

Dose of vincristine mg/kg body wt.	Mean no. of metaphases ± SD			
	Subscapular cortex	Mid-cortex	Cortex/ medulla	Medulla
2	32.7 ± 10.6 (3.3)	41.0 ± 7.2 (2.3)	14.4 ± 8.2 (2.6)	2.7 ± 1.7 (0.5)
3	22.5 ± 9.8 (3.1)	25.4 ± 8.4 (2.7)	12.1 ± 8.2 (2.6)	5.2 ± 1.9 (0.6)
4	24.9 ± 7.6 (2.4)	23.9 ± 9.3 (2.9)	18.6 ± 3.7 (1.2)	5.2 ± 2.3 (0.7)
5	24.3 ± 7.7 (2.4)	18.1 ± 5.6 (1.8)	8.0 ± 3.4 (1.1)	3.1 ± 2.6 (0.8)

Sample (N) = 10
Nos. in parenthesis = SE
No significant differences between doses in subscapular region ($p > 0.05$)

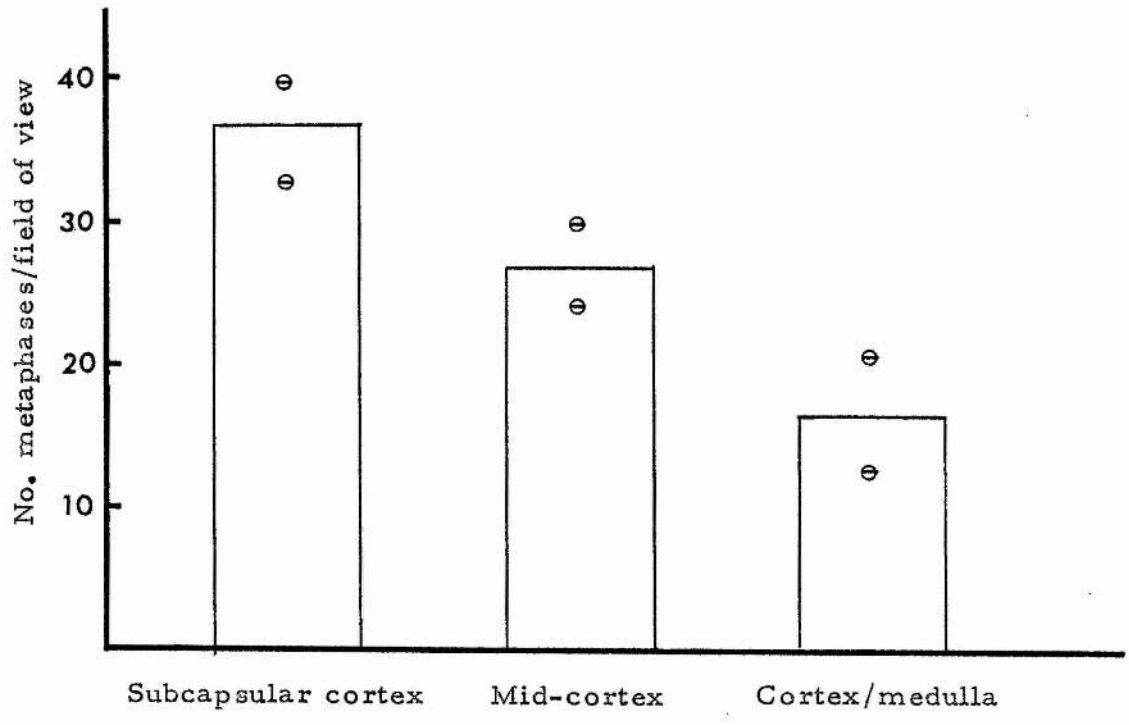
Vincristine dose response: Preliminary study

Fig. (22)



Distribution of metaphases in thymus: Preliminary study

Fig. (23)



of the thymus: outer, mid and inner cortex was determined. Table (21) and Fig. (23) show that there are more proliferating cells in the outer and less in the inner cortex. In a further study with vincristine at doses of 2-5 mg/kg, the no. of dividing cells in the three cortical regions as well as in the medulla were enumerated. Table (22) and Fig. (24) show that mitotic cells were most frequent in the outer and mid-cortex, with less in the inner cortex and negligible cell proliferation in the medulla at all doses tested. In the subcapsular cortex, where most cell division occurred, there were no significant differences with different doses of vincristine, therefore the lowest effective dose ie. 2 mg was chosen as optimal for further experiments.

In a time study between 1-6 h after vincristine treatment at the optimal dose (2 mg/kg), the accumulation of arrested mitotic nuclei in the various regions of the thymic cortex was quantitated. In this and subsequent experiments, the no. of dividing cells/grid area were determined. Table (23) and Fig. (25) show that in the cortical regions, where cell proliferation is most apparent, uniform increases in the no. of dividing cells with time are seen between 1-3 h after treatment with vincristine. Therefore an additional time study for a duration of 3 h with shorter intervals was undertaken in normal mice. Table (24) and Fig. (26) show the data from this experiment. As seen previously, cell proliferation is greatest in the subcapsular cortex and least in the cortex/medulla. The no. of mitotic cells increases uniformly with time in all regions of the thymic cortex. A similar study was carried out in pregnant animals at 15 days of gestation (Table 25 and Fig. 27). Note the

Cell proliferation in different thymic regions:

Vincristine dose study

Fig. (24)

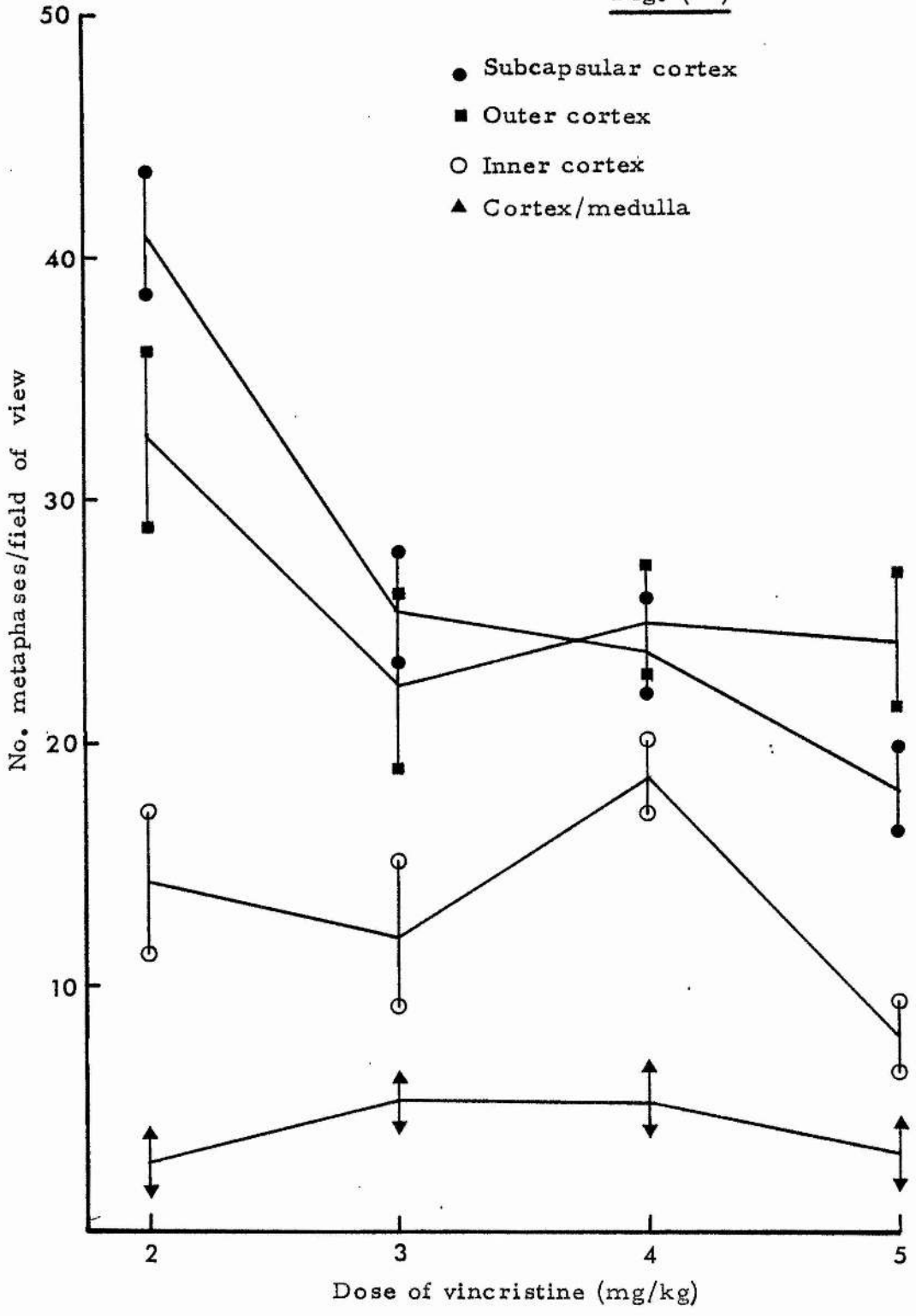


Table (23). Accumulation of mitotic cells: time study in non-pregnant mice

Time after vincristine treatment (hours)	Mean no. of metaphases ± SD			
	Subscapular cortex	Outer cortex	Inner cortex	Cortex/ medulla
1	6.8 ± 2.9 (0.9)	3.2 ± 2.3 (0.7)	1.8 ± 1.4 (0.4)	1.3 ± 1.3 (0.4)
2	9.2 ± 3.6 (1.1)	5.3 ± 2.7 (0.9)	2.8 ± 2.1 (0.7)	1.7 ± 1.6 (0.5)
3	14.7 ± 3.8 (1.2)	6.1 ± 4.9 (1.5)	5.7 ± 3.9 (1.2)	6.6 ± 3.0 (0.9)
4	12.1 ± 2.6 (0.8)	5.2 ± 3.2 (1.0)	4.8 ± 3.2 (1.0)	4.4 ± 3.8 (1.2)
5	10.9 ± 5.7 (1.0)	8.2 ± 5.8 (1.8)	5.4 ± 3.1 (1.0)	2.9 ± 1.9 (0.6)
6	16.2 ± 4.0 (1.3)	9.2 ± 4.8 (1.5)	7.4 ± 5.0 (1.6)	4.6 ± 2.6 (0.8)

Sample (N) = 10
Nos. in parenthesis = SE
Highly significant differences in nos. of mitotic cells between extremes of the cortex ($p < 0.001$)

Table (24). Accumulation of mitotic cells: time study in non-pregnant mice

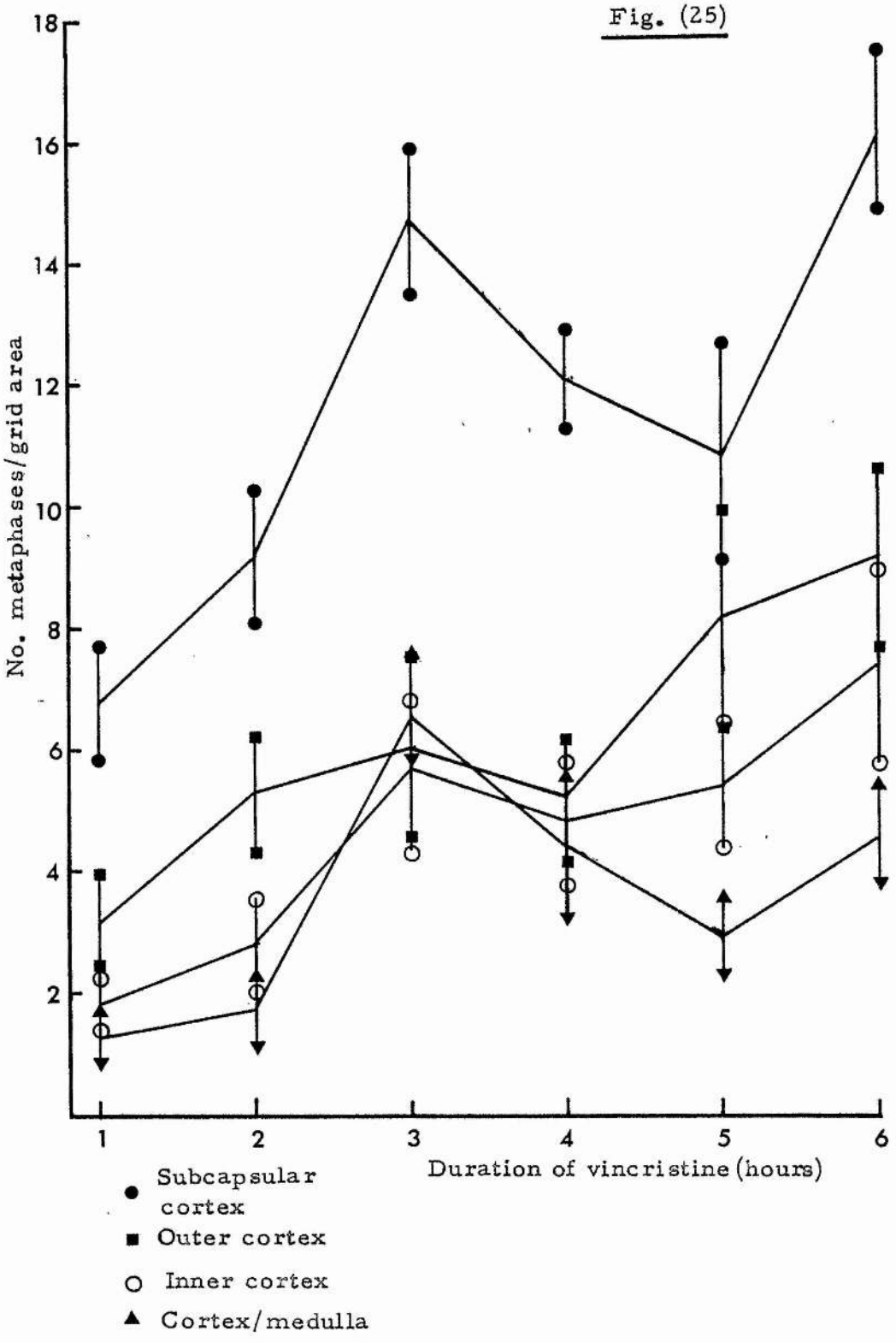
Time after vincristine treatment (hours)	Mean no. of metaphases ± SD			
	Subscapular cortex	Outer cortex	Inner cortex	Cortex/ medulla
0.5	4.2 ± 1.8 (0.6)	2.3 ± 1.4 (0.4)	0.8 ± 0.8 (0.3)	0.7 ± 1.9 (0.6)
1.0	6.5 ± 2.9 (0.9)	3.6 ± 2.1 (0.7)	2.1 ± 1.9 (0.6)	1.1 ± 1.0 (0.3)
1.5	8.4 ± 4.1 (1.3)	4.1 ± 2.7 (0.9)	2.7 ± 2.3 (0.7)	1.3 ± 1.4 (0.4)
2.0	10.8 ± 4.6 (1.5)	5.5 ± 3.2 (1.0)	3.5 ± 2.4 (0.8)	1.6 ± 1.6 (0.5)
2.5	14.2 ± 5.4 (1.7)	6.3 ± 3.3 (1.0)	4.6 ± 3.1 (1.0)	2.8 ± 1.9 (0.5)
3.0	16.1 ± 5.9 (1.9)	7.0 ± 4.0 (1.3)	5.8 ± 3.8 (1.2)	2.7 ± 1.9 (0.6)

Sample (N) = 10
Nos. in parenthesis = SE

Cell proliferation in thymic cortex:

Time study in normal mice

Fig. (25)



Cell proliferation in thymic cortex:

Time study in normal mice

Fig. (26)

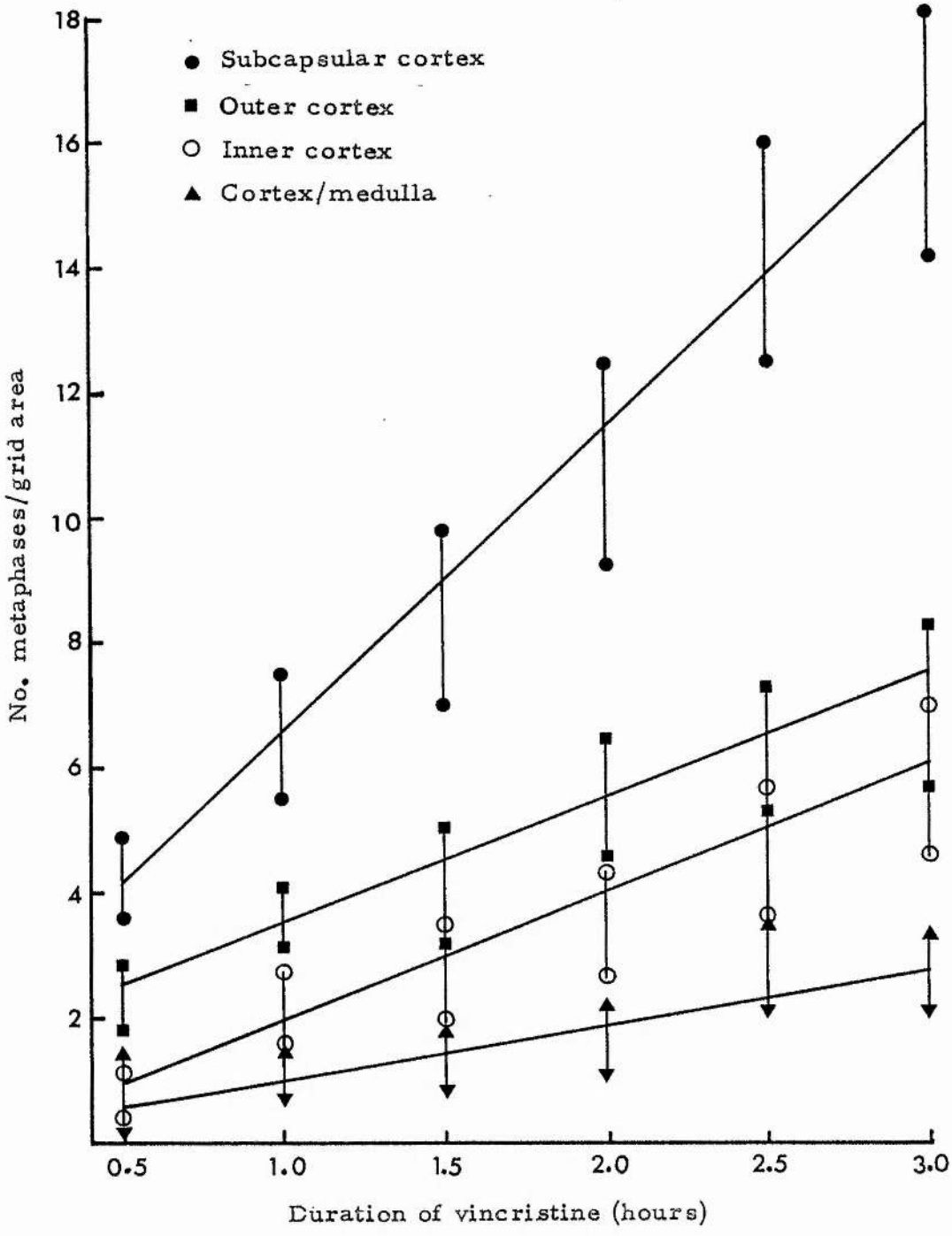


Table (25). Accumulation of mitotic cells: time study in pregnant mice

Time after vincristine treatment (hours)	Mean no. of metaphases ± SD			
	Susbscapular cortex	Outer cortex	Inner cortex	Cortex/ medulla
0.5	3.5 ± 1.9 (0.6)	2.3 ± 1.7 (0.5)	1.4 ± 1.2 (0.4)	1.1 ± 1.2 (0.4)
1.0	5.2 ± 2.3 (0.7)	2.5 ± 1.7 (0.5)	1.5 ± 2.0 (0.6)	1.1 ± 1.2 (0.4)
1.5	5.4 ± 2.0 (0.6)	2.5 ± 1.6 (0.5)	1.4 ± 1.2 (0.4)	1.0 ± 1.2 (0.4)
2.0	5.5 ± 2.6 (0.8)	3.2 ± 2.1 (0.7)	1.3 ± 1.5 (0.5)	1.2 ± 1.4 (0.4)
2.5	6.8 ± 3.1 (1.0)	3.4 ± 2.5 (0.8)	2.0 ± 1.7 (0.2)	2.0 ± 1.7 (0.5)
3.0	7.9 ± 3.6 (1.1)	3.9 ± 1.1 (0.3)	2.4 ± 2.4 (0.8)	1.8 ± 1.9 (0.6)

Sample (N) = 10

Table (26). Cellularity of thymus in histological sections

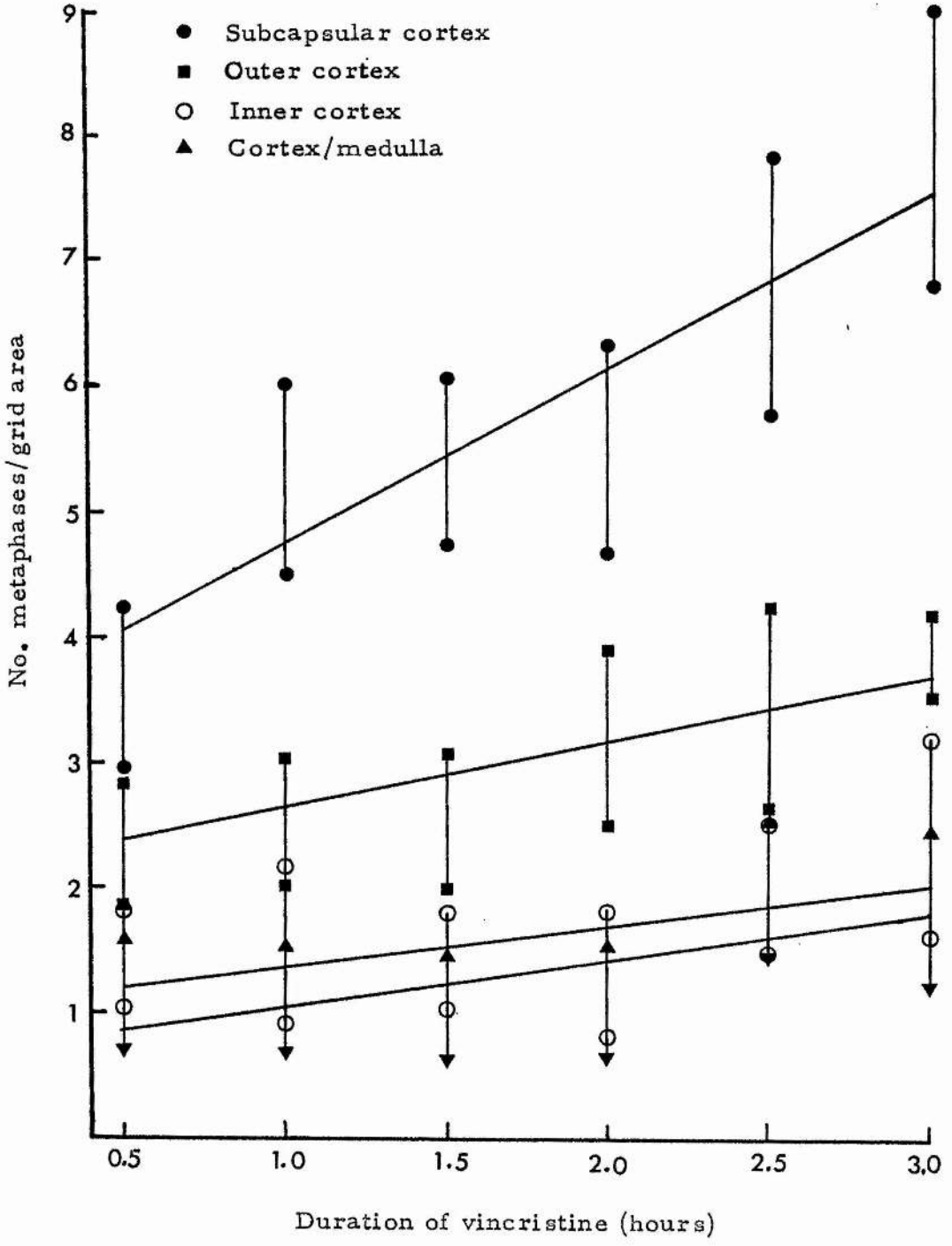
	Mean no. of cells ± SD			
	Subscapular cortex	Outer cortex	Inner cortex	Cortex/ medulla
Normal non-pregnant	131.9 ± 20.1	152.1 ± 31.2	149.5 ± 12.9	119.5 ± 21.0
Pregnant	117.3 ± 17.4	115.9 ± 14.8	102.4 ± 18.2	91.3 ± 19.0

Sample (N) = 10

Cell proliferation in thymic cortex:

Time study in pregnant mice

Fig. (27)



scales on the y-axis are different in this figure. The accumulation of arrested, dividing cells shows a similar profile with increasing time to that in normal animals, although the no. of dividing cells are very much less in the pregnant animals.

The total no. of cells in the various thymic regions in normal and pregnant animals was determined (Table 26). The average diameters of metaphase and interphase nuclei in normal and pregnant animals was also measured, the data is shown in Table (27). An average of 5.25 microns was taken to represent the diameter of interphase nuclei in both normal and pregnant animals and the diameter of metaphase nuclei was found to be 4.0 microns in both groups of animals.

The data from the previous two kinetic studies (Tables 24 and 25) were used to calculate the cell production rates in different regions of the thymic cortex in normal and pregnant animals, as described previously. In normal animals, the rate of cell production is maximal at 40/1000 cells entering mitosis/h in the subcapsular cortex, the rate diminishes to 21/1000/h in the inner cortex (Figs. 28-31). During pregnancy, the subcapsular cortex is also the site of greatest cell proliferation, but at a much diminished rate of 13.5/1000/h (Figs. 32-35). The differences are most pronounced in the inner and mid-cortex where the cell production rates are 5.5 and 3.7 respectively. The differences are negligible in the cortex/medulla, the cell production rates being uniformly low at 3.3 and 3.0/1000/h in normal and pregnant animals respectively. If it is assumed that cell proliferation is confined to the cortex proper and negligible in the cortex/medulla under normal conditions, then the rate of cell production is found to be reduced during

Table (27). Diameters of metaphase and interphase nuclei

	Diameter ± SD (microns)
Interphase nuclei (normal animals)	5.3 ± 0.8
Interphase nuclei (pregnant animals)	5.2 ± 0.7
Metaphase nuclei	4.0 ± 0.3

Sample (N) = 200

Table (28). Change in weight of thymus and growth of animals during experimental period

Time (weeks)	Wt. of thymus ± SD (mg)	SE	Wt. of animals ± SD (g)	SE
0	81.8 ± 19.8	6.2	22.5 ± 1.0	0.3
1	74.2 ± 20.3	6.4	24.3 ± 2.2	0.7
2	58.7 ± 13.5	4.3	26.3 ± 2.2	0.7
3	58.5 ± 12.6	4.0	28.3 ± 1.4	0.4
4	55.2 ± 6.0	1.9	29.9 ± 3.5	1.3
5	53.2 ± 11.9	3.8	30.6 ± 2.3	0.7
6	45.9 ± 14.6	5.5	31.6 ± 2.0	0.6

Sample (N) = 10

No significant involution in thymus between 2-6 weeks ($p > 0.05$)

Difference in weight of animals between 0-6 weeks ($p < 0.001$)

Cell production rate in thymic cortex: Normal mice

Fig. (28)

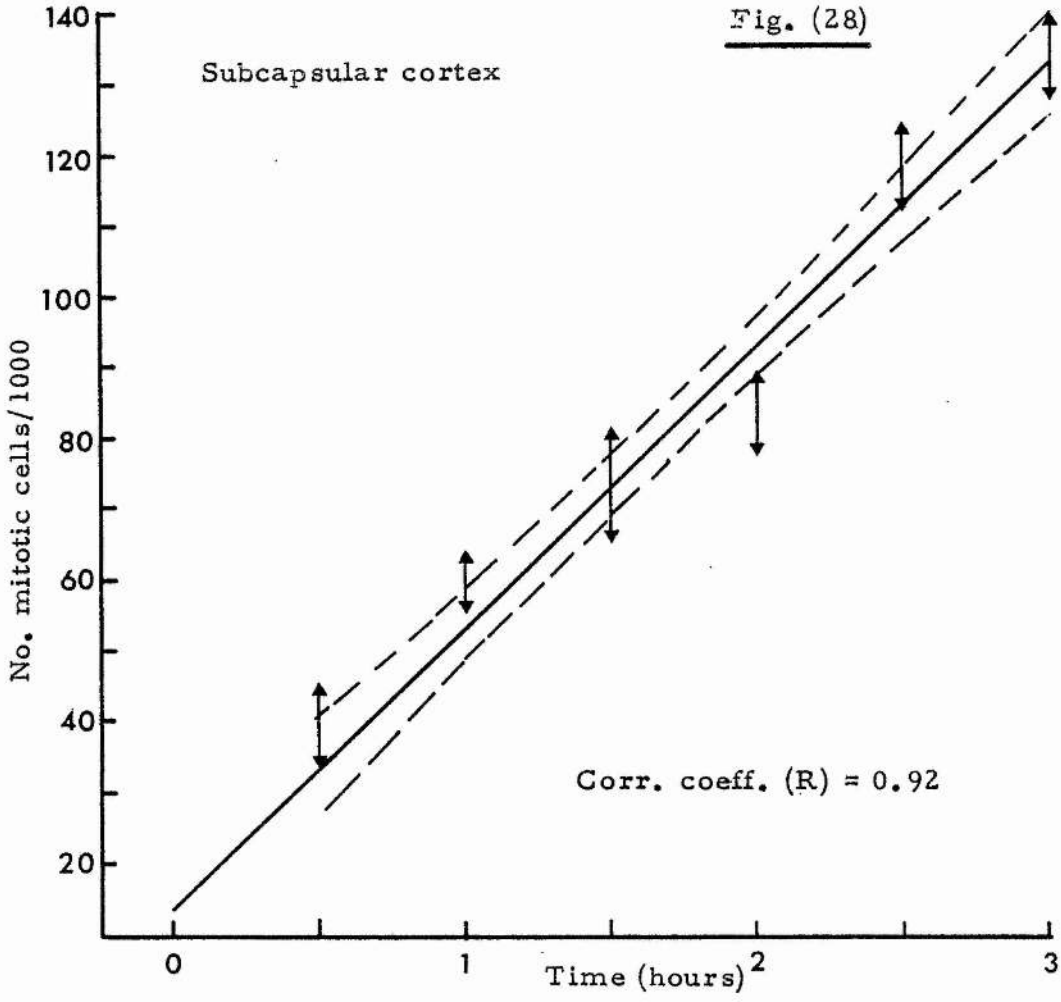
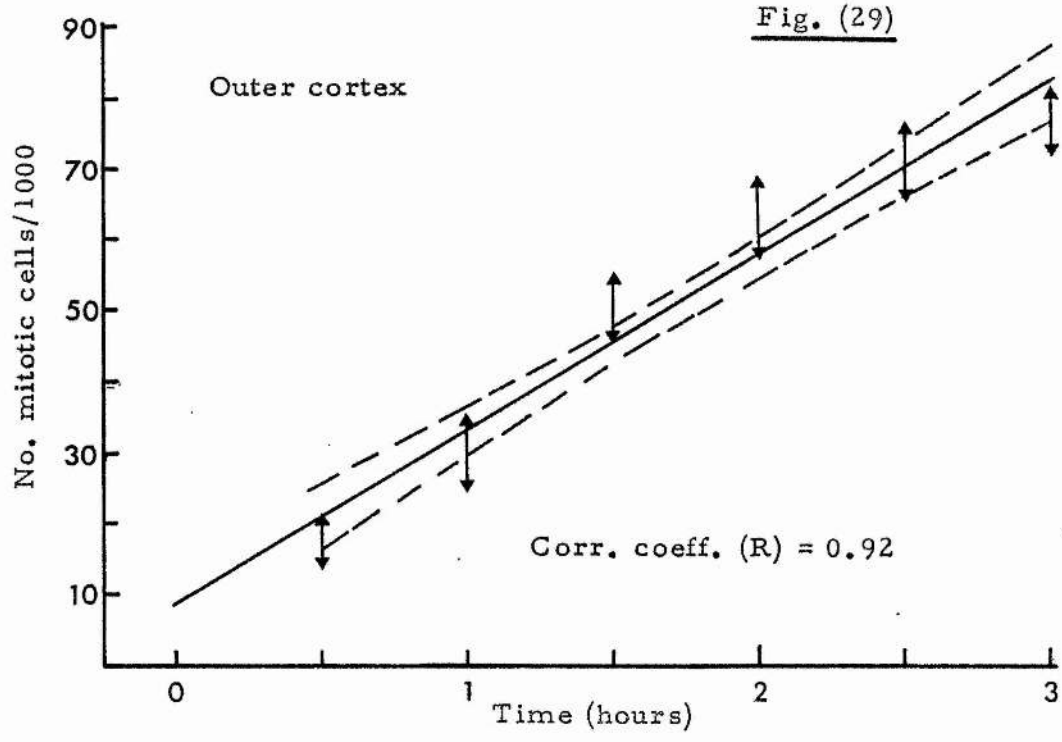
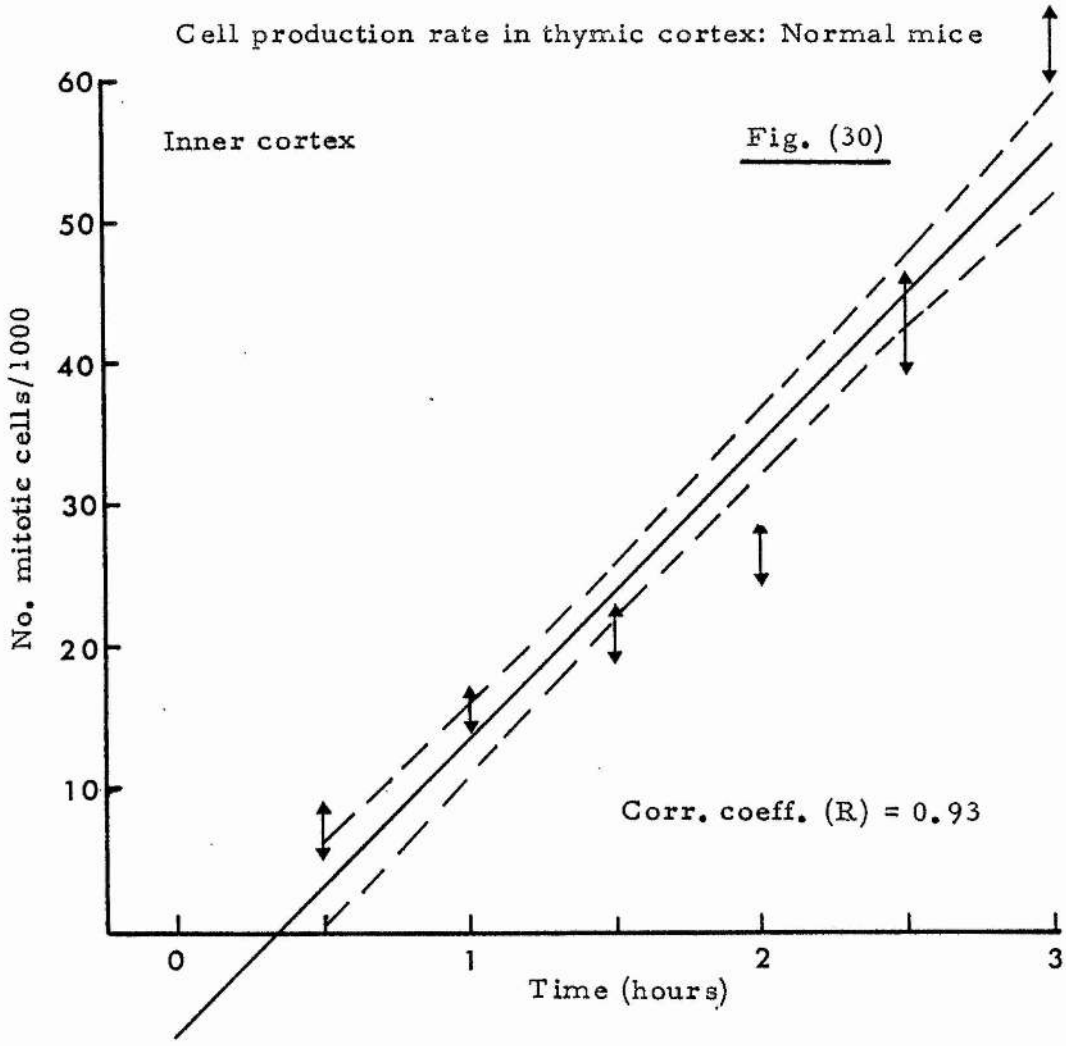
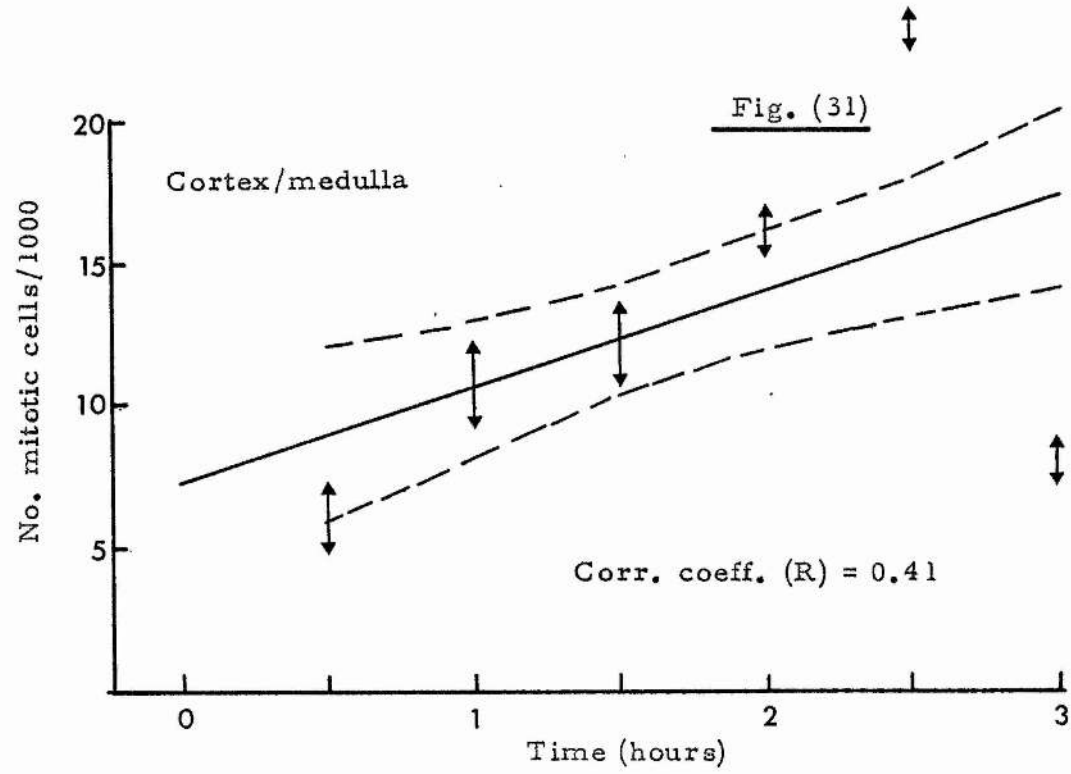


Fig. (29)



Cell production rate in thymic cortex: Normal mice





Cell production rate in thymic cortex: Pregnant mice

Fig. (32)

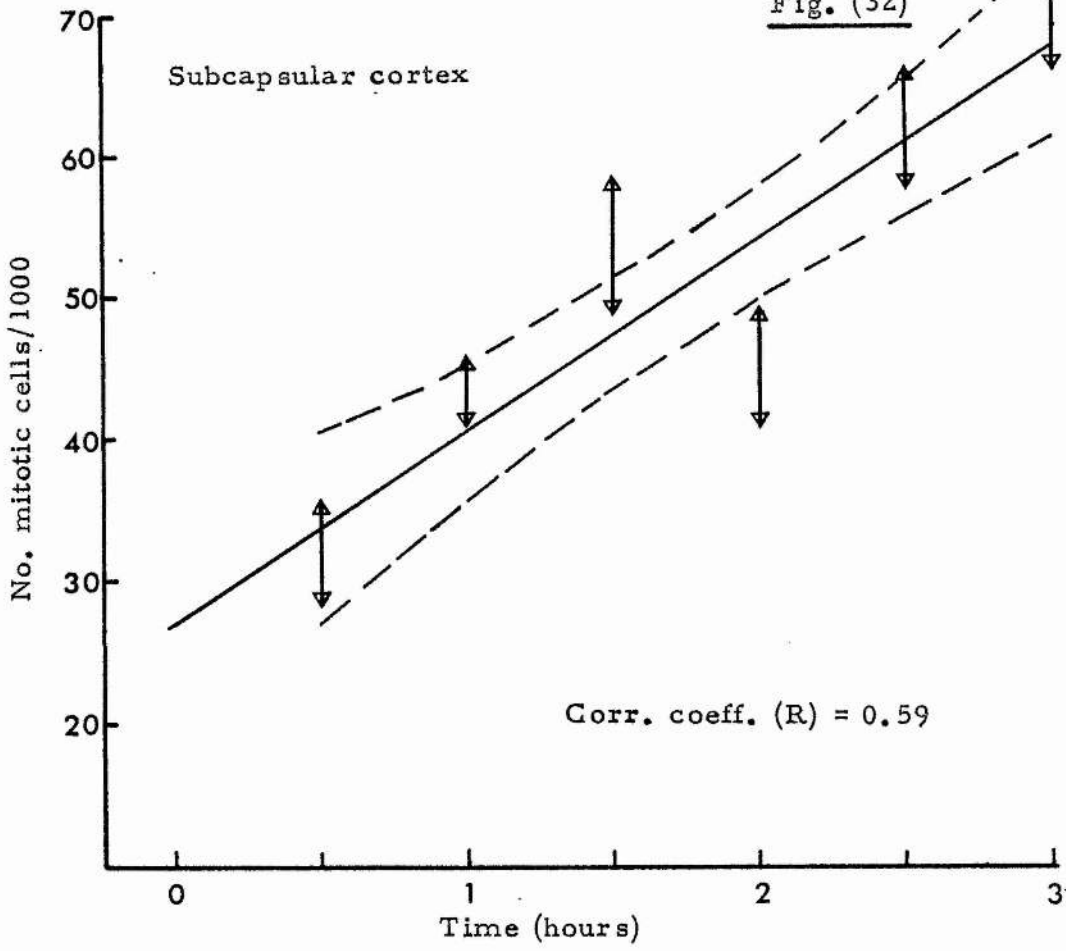
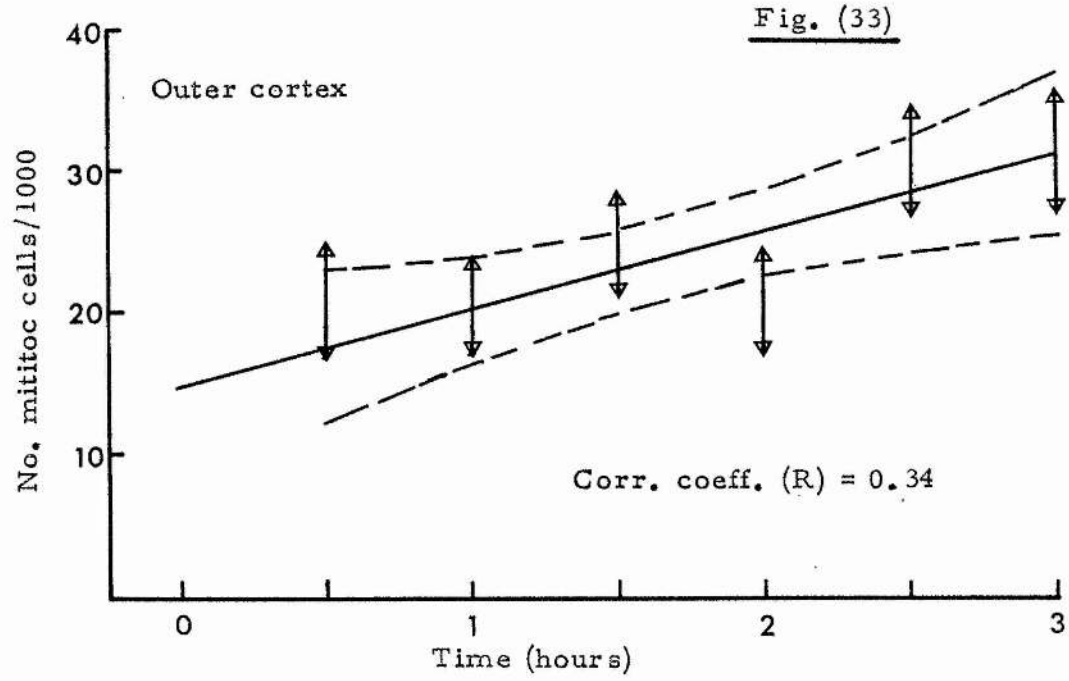
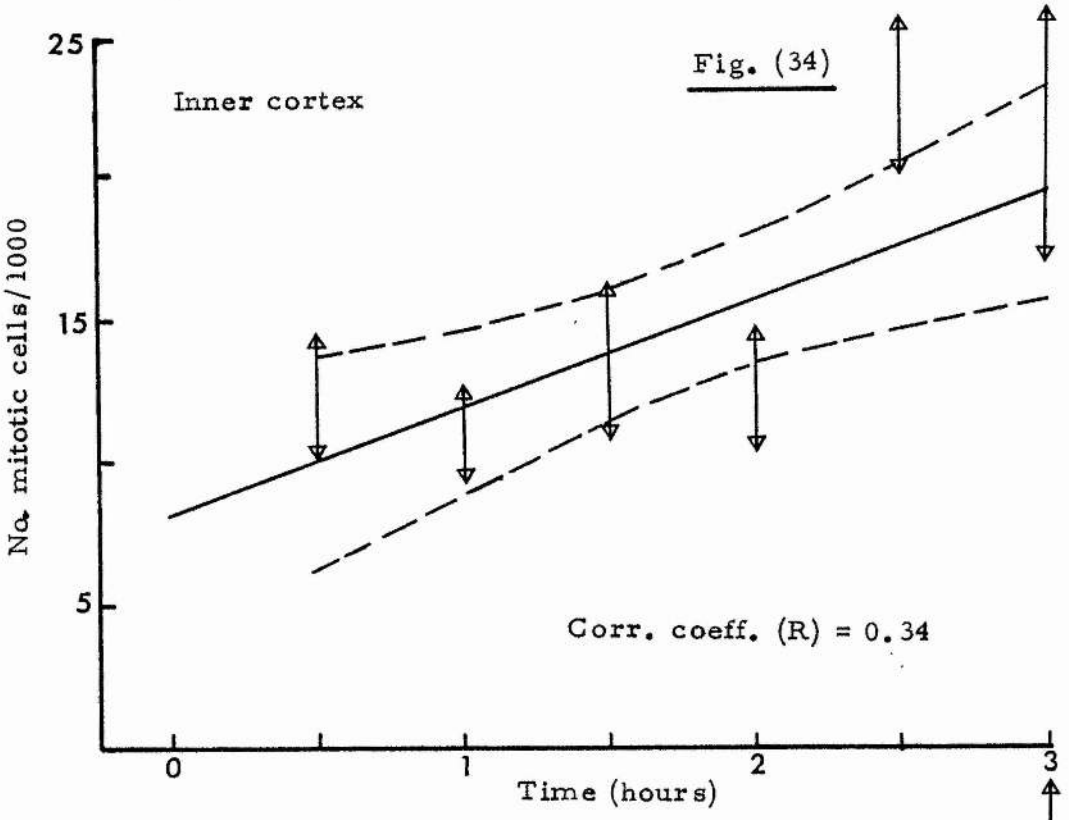
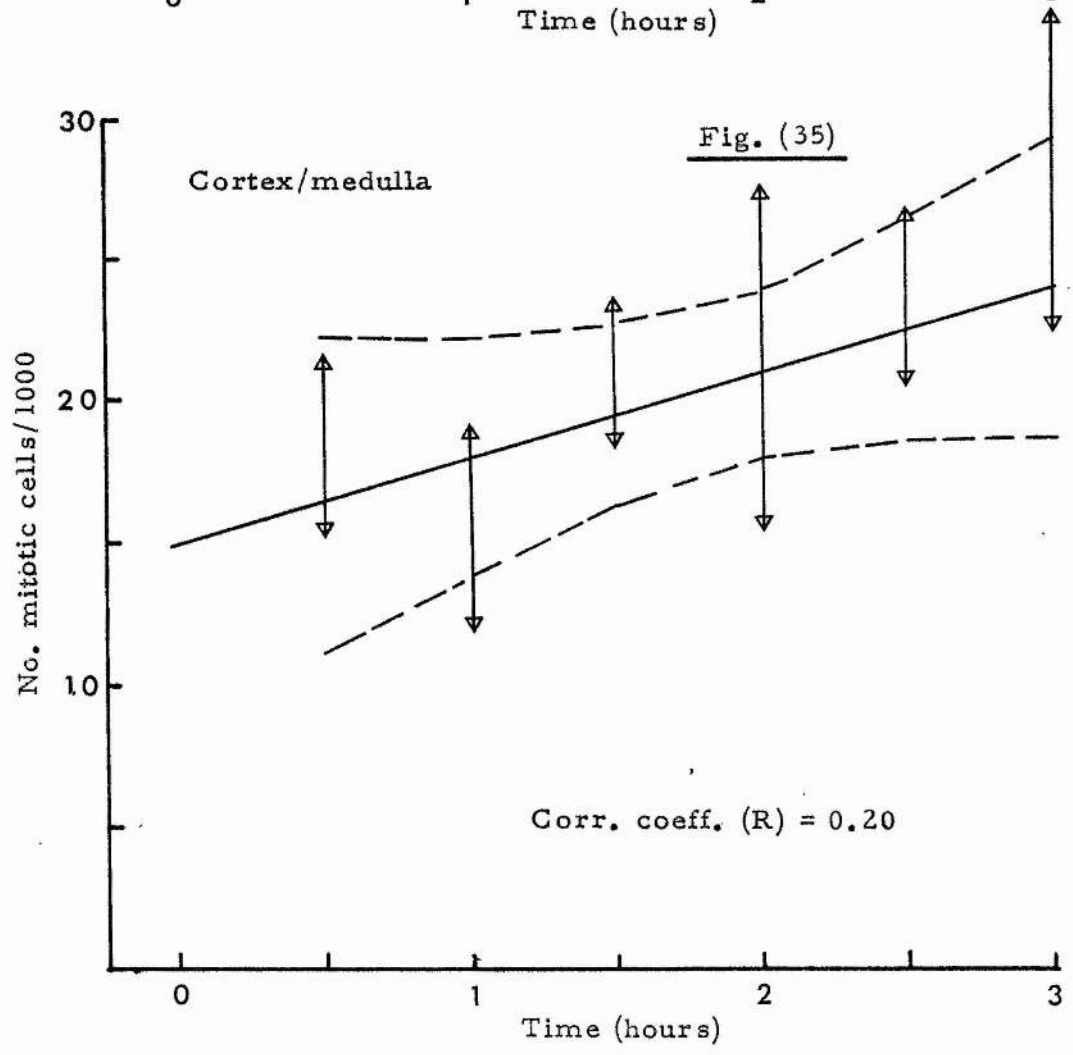


Fig. (33)



Cell production rate in thymic cortex: Pregnant mice





pregnancy to approx. 25% of the normal rate. The data from this study is summarised in Table (29) and includes the turnover time and growth fraction obtained from these findings. These values were derived by assuming an average cell cycle time of 9.0 h for thymus lymphocytes, a value obtained by most other investigators (Metcalf, 1964; Fabrikant and Foster, 1971; Bryant, 1972; and Zaitoun et al, 1979). The turnover time was calculated for 1000 cells, from the cell production rate (no. of dividing cells/1000 cells/h); the growth fraction being derived from the ratio of the cell cycle time/turnover time.

An interval of up to 4 weeks generally elapsed from the time of arrival of the mice from the suppliers till late gestation when they were sacrificed. The natural decrease in the thymus weight at weekly intervals for a period of up to 6 weeks after arrival was investigated. In addition, the animals were weighed at these times to ensure that growth proceeded normally. The thymic weight decreased fairly rapidly within the first 2 weeks, after which the decrease was more gradual. The health of the animals was evidently not impaired as judged by their increasing body weight over the experimental period (Table 28 and Fig. 36).

C. PHA responsiveness during pregnancy

In a preliminary study, the PHA responsiveness of lymph node and spleen cells were compared from a pregnant animal at different cell concentrations. At the lower cell concentrations, lymph node cells were more responsive than the spleen cells (Table 30 and Figs. 37 and 38).

Table (29). Summary of data from cytokinetic study of thymus

Thymic region	Non-pregnant			Pregnant			Decrease in cell production rate during pregnancy (%) *
	Cell production rate (per 1000 cells/h)	Growth fraction (%)	Turnover time (h)	Cell production rate (per 1000 cells/h)	Growth fraction (%)	Turnover time (h)	
Subscapular cortex	40.0	36.0	25.0	13.5	12.1	74.1	33.8
Outer cortex	25.0	22.5	40.0	5.5	4.9	182.0	19.6
Inner cortex	21.0	18.9	47.6	3.7	3.3	270.0	17.6
Cortex/medulla	3.3	2.9	303.0	3.0	2.7	333.3	90.9

* Refers to cell production rates during pregnancy as a % of the normal rate

Average decrease excluding cortex/medulla region = 23.6 ($\pm$ 8.8)%

Change in size of thymus and growth of mice during experimental period.

Fig. (36)

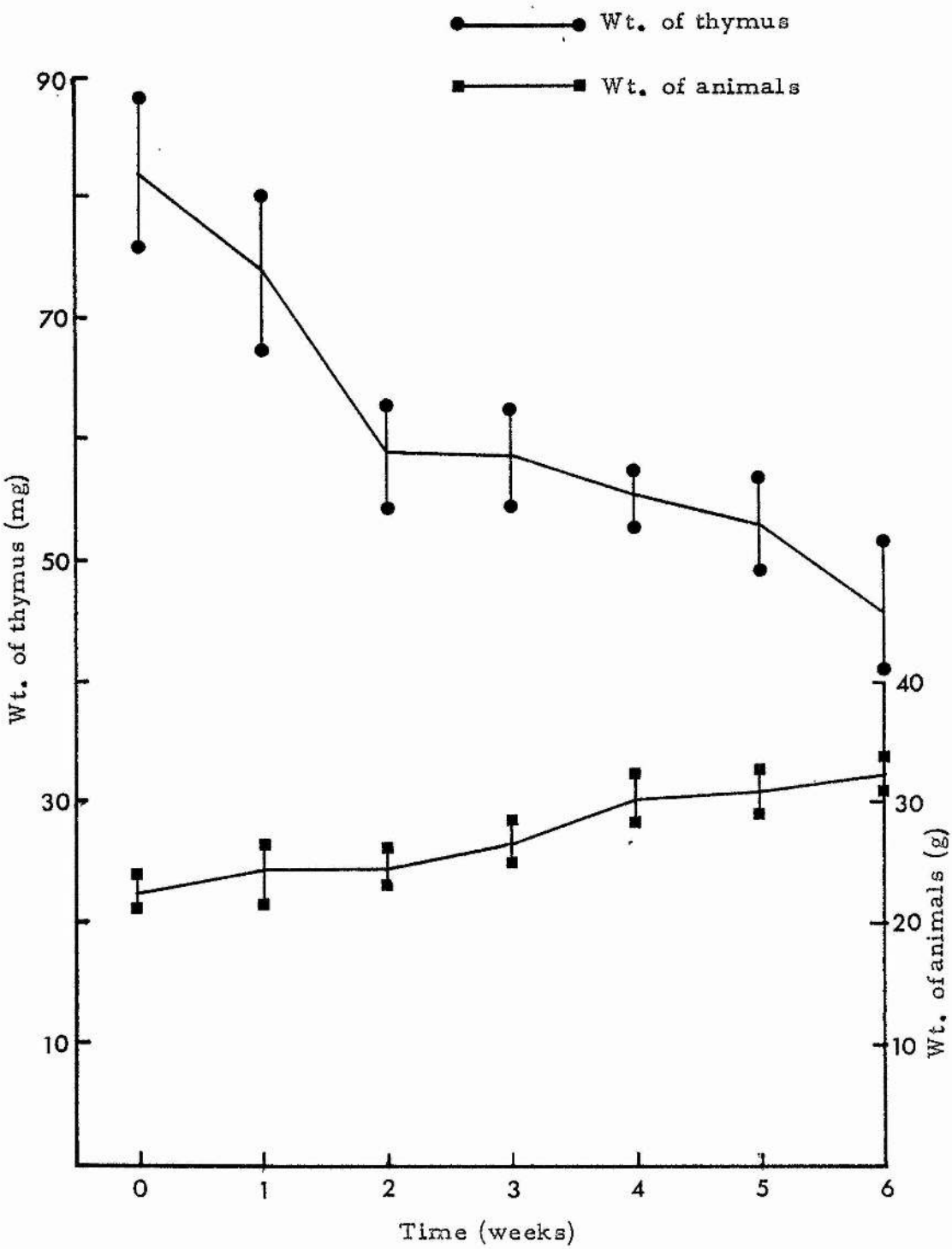


Table (30). PHA response of spleen and lymph node cells during pregnancy : Expt. (1)

Cells	No. cells/well	PHA	Mean cpm ± SD	SI ± SE	N
Spleen	10^6	+	1279 ± 392	20.0 ± 3.1	6
"	"	-	64 ± 25		"
"	5×10^5	+	661 ± 221	12.2 ± 2.6	"
"	"	-	54 ± 22		"
"	2×10^5	+	544 ± 172	9.5 ± 1.9	"
"	"	-	57 ± 20		"
Lymph node	10^6	+	1500 ± 464	25.4 ± 7.5	4
"	"	-	59 ± 28		"
"	5×10^5	+	1208 ± 223	16.5 ± 2.5	"
"	"	-	73 ± 16		"
"	2×10^5	+	895 ± 123	14.7 ± 2.6	"
"	"	-	61 ± 20		"

Difference between lymph node and spleen cells at higher cell concs. ($p < 0.01$).

⊖ PHA responsiveness of spleen and lymph node cells during pregnancy

Fig. (37).

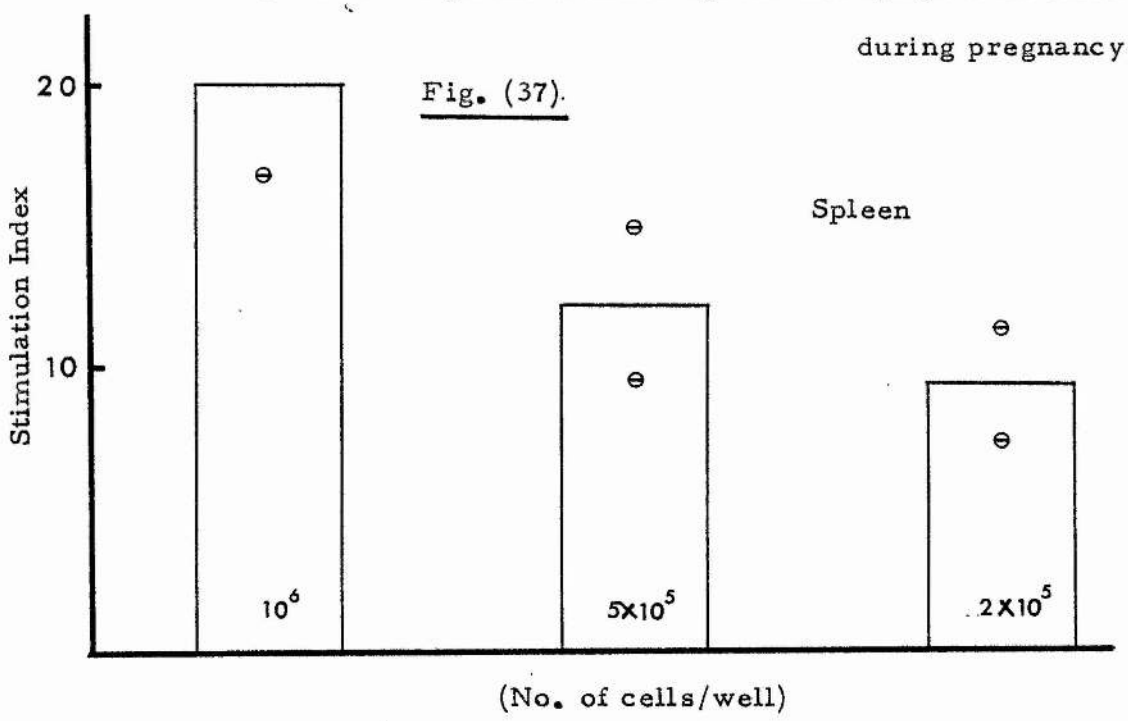
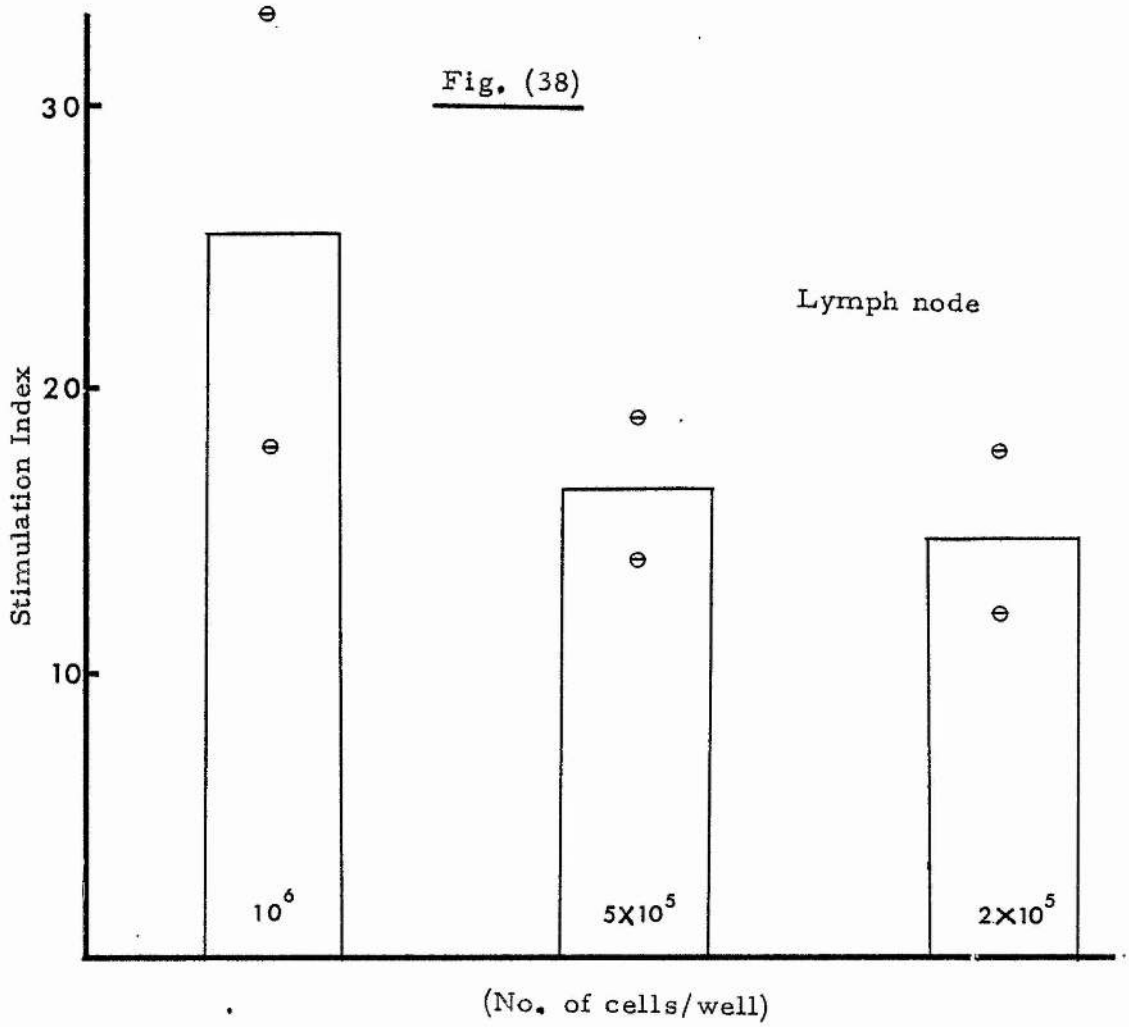


Fig. (38)



In a more extensive study, the PHA response of spleen and lymph node cells at different cell concentrations from pregnant, normal and young virgin female mice was investigated. Higher cell concentrations did not elicit greater proliferative responses and in most cases were less than the cells at more lower concentrations. Both lymph node and spleen cells from the adult animal showed greater stimulation than in the pregnant animal, the lymph node cells being more responsive in the normal animal. Lymph node and spleen cells were stimulated to a similar extent in the pregnant animal. Both groups of cells from the young animal did not differ much in their PHA responsiveness and generally responded slightly less than the adult animal. Its lymph node cells were stimulated to approx. the same extent as lymph node cells from the pregnant animal, whereas the spleen cells were more responsive at the higher cell concentration (Table 31 and Figs. 39-41). A summary of the differential cell counts performed on the spleens from the animals used in this experiment is shown in Table (33). It can be seen that during pregnancy, the lymphocyte count is diminished compared to the normal adult and young animals. In contrast, erythroid cells are more abundant in the pregnant spleen.

A similar experiment was carried out with pregnant and normal adult animals with lower cell concs., at 10^6/well they were not found to be maximally stimulated in the previous experiments. In the pregnant animal, at both cell concentrations, lymph node cells were stimulated to a greater extent than spleen cells; less pronounced differences exist in the normal animal (Table 32 and Figs. 42 and 43). In the summarised differential cell count data in Table (33), it can

Table (31). PHA responses of normal and pregnant mice: Expt. (2)

Cells	No. cells/ well	PHA	Mean cpm ± SD	SI ± SD	p
Pregnant spleen	10^6	+	825 ± 197	12.7 ± 2.2	
"	"	-	65 ± 23		
"	5×10^5	+	680 ± 204	14.4 ± 3.5	
"	"	-	47 ± 25		
Pregnant lymph node	10^6	+	1500 ± 343	20.0 ± 2.6	
"	"	-	75 ± 18		
"	5×10^5	+	1796 ± 404	20.2 ± 3.5	
"	"	-	89 ± 34		
Adult spleen	10^6	+	842 ± 137	18.3 ± 2.6	< 0.001
"	"	-	46 ± 16		
"	5×10^5	+	1453 ± 185	18.4 ± 1.9	< 0.01
"	"	-	79.6 ± 16		
Adult lymph node	10^6	+	1266 ± 152	24.8 ± 3.6	< 0.01
"	"	-	51 ± 18		
"	5×10^5	+	2091 ± 453	33.1 ± 6.0	< 0.001
"	"	-	63 ± 25		
Young spleen	10^6	+	1662 ± 815	21.9 ± 5.1	< 0.001
"	"	-	76 ± 21		
"	5×10^5	+	659 ± 218	14.0 ± 3.2	> 0.1
"	"	-	47 ± 23		
Young lymph node	10^6	+	1931 ± 322	24.4 ± 5.5	< 0.05
"	"	-	79 ± 41		
"	5×10^5	+	1783 ± 411	21.0 ± 3.4	> 0.1
"	"	-	85 ± 27		

Sample (N) = 6

Tabulated p values compare pregnant and normal animals

Significant differences between pregnant spleen and lymph node cells at all cell concs. ($p < 0.001$); differences in normal animals only at lower cell concs.

PHA responsiveness in normal and pregnant mice

Fig. (39)

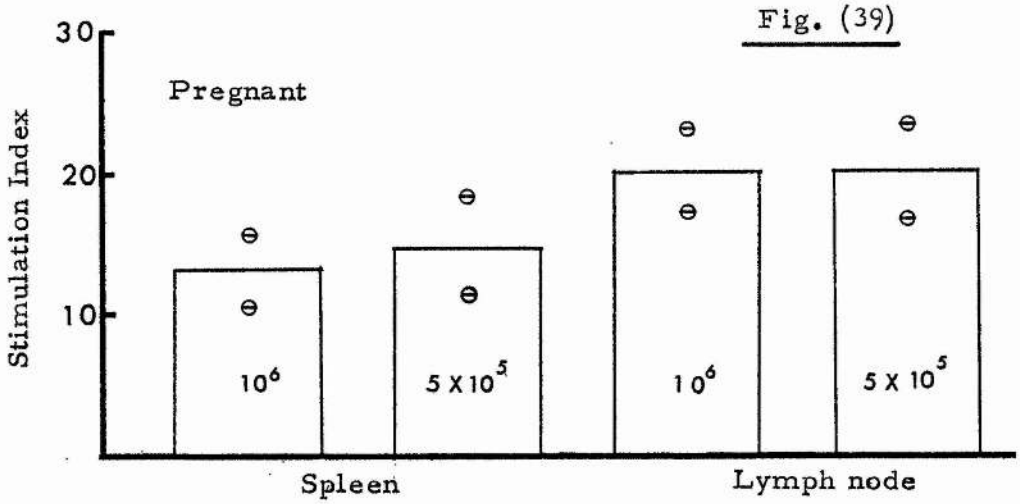


Fig. (40)

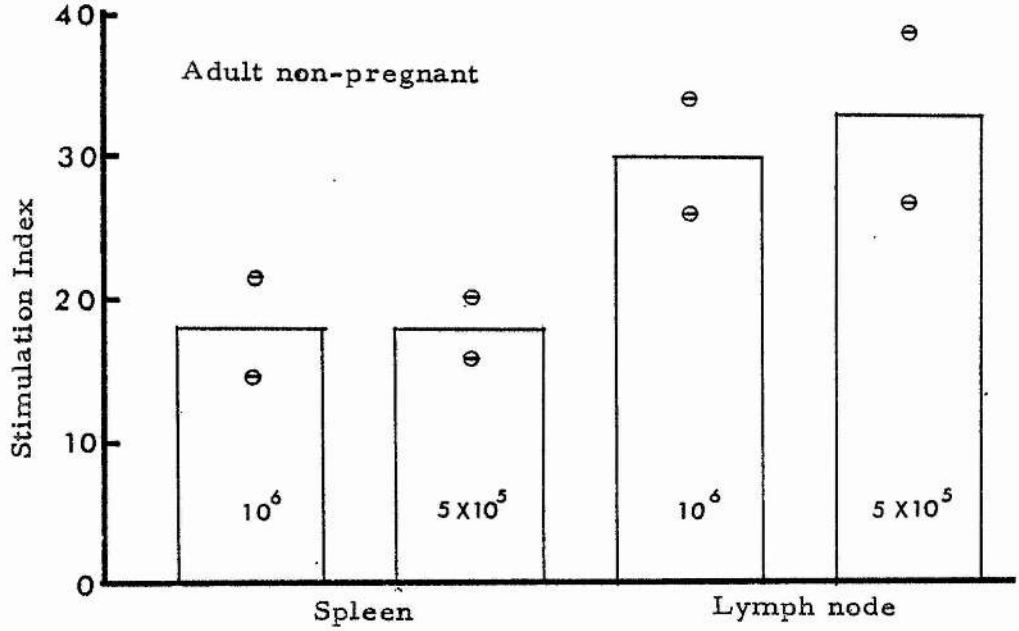


Fig. (41)

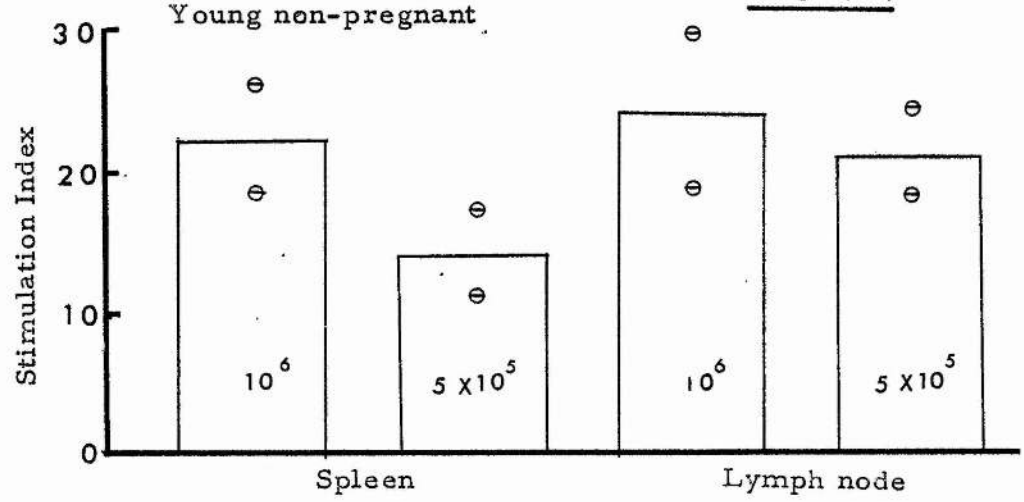


Table (32). PHA responses of normal and pregnant mice: Expt. (3)

Cells	No. cells/ well	PHA	Mean cpm ± SD	SI ± SD	p
Pregnant spleen	5×10^5	+	2217 ± 531	15.7 ± 1.9	> 0.05
"	"	-	141 ± 25		
"	2×10^5	+	2182 ± 982	16.8 ± 3.3	> 0.1
"	"	-	130 ± 22		
Pregnant lymph node	5×10^5	+	2400 ± 634	25.8 ± 3.6	< 0.001
"	"	-	93 ± 20		
"	2×10^5	+	2565 ± 684	27.3 ± 3.8	< 0.001
"	"	-	94 ± 19		
Normal spleen	5×10^5	+	1721 ± 452	14.2 ± 2.0	
"	"	-	121 ± 27		
"	2×10^5	+	1684 ± 214	15.3 ± 1.8	
"	"	-	110 ± 28		
Normal lymph node	5×10^5	+	1927 ± 523	17.1 ± 2.5	
"	"	-	113 ± 28		
"	2×10^5	+	1405 ± 154	18.2 ± 3.2	
"	"	-	77 ± 31		

Sample (N) = 6

Tabulated p values refer to comparisons between pregant and normal animals

Difference between pregnant spleen and lymph node cells ($p < 0.001$); difference in normal animal is less ($p < 0.01$ and 0.02).

PHA responsiveness in normal and pregnant mice

Fig. (42)

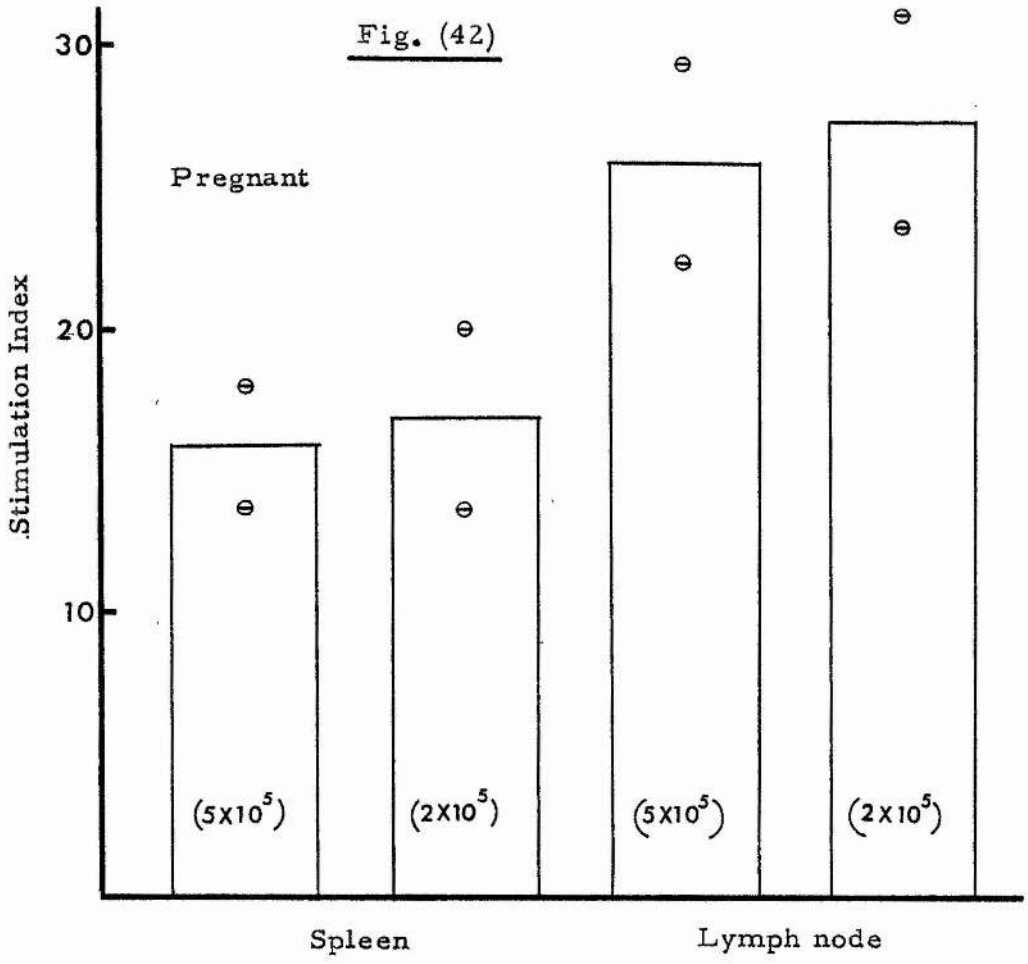


Fig. (43)

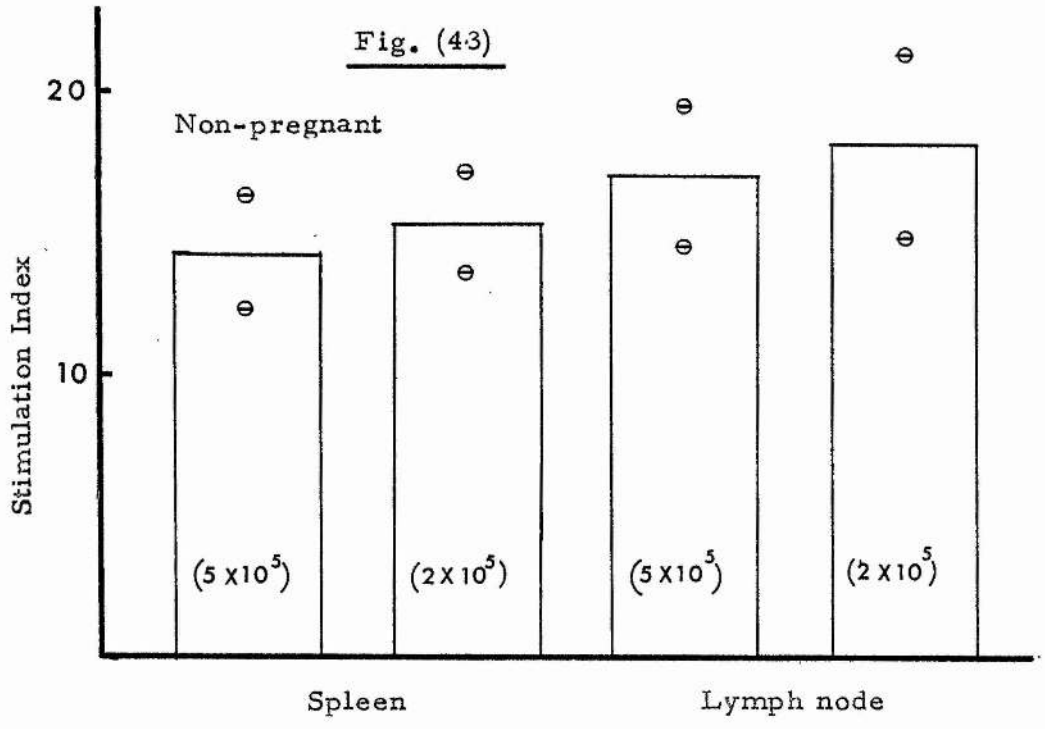


Table (33). Summary of differential cell counts on spleen

Expt. (2)

	Proportion of cells (%) in spleen		
	Pregnant	Adult, non-pregnant	Young, non-pregnant
Lymphocytes	20.9	60.4	40.8
Erythroid	29.1	5.2	8.4
Myeloid	3.7	4.8	7.6
Monocytes	-	2.4	5.2
Damaged/unclassified	8.2	7.6	12.4
Tissue cells	35.2	14.4	19.2
Blast cells, plasma cells and macrophages	2.8	5.6	6.4

Expt. (3)

Lymphocytes	14.6	67.2
Erythroid	41.3	0.8
Myeloid	6.7	2.8
Monocytes	4.6	2.0
Damaged/unclassified	2.2	7.6
Tissue cells	27.0	14.0
Blast cells, plasma cells and macrophages	3.8	5.6

be seen that the spleen of the pregnant animal has a much reduced proportion of lymphocytes than the non-pregnant control. Furthermore, the proportion of erythroid cells in the spleen is very much greater in the pregnant animal.

D. Extra-medullary haemopoiesis during pregnancy

The amount of ^{59}Fe activity recovered in the spleens and femurs of pregnant and normal animals previously treated with the isotope was assayed. Table (34) shows the data from this experiment. There are no significant differences in the amount of isotope incorporated/mg of spleen tissue; however the spleens in pregnant mice incorporated more ^{59}Fe *per se*. There was no significant difference in the activity in femurs between the groups.

Table (34). Summary of data from ^{59}Fe studies in vivo

	Pregnant	Non-pregnant	p
Mean wt. of spleen (mg)	84.9 ± 22.8	65.2 ± 8.8	< 0.01
^{59}Fe activity/spleen (cpm)	1619.9 ± 574.8	898.5 ± 225.9	< 0.02
^{59}Fe activity/spleen tissue (cpm/mg)	19.0 ± 8.4	13.7 ± 3.9	> 0.1
Recovery of ^{59}Fe in spleen (%)	2.1 ± 0.8	1.2 ± 0.3	< 0.05
^{59}Fe activity/femur (cpm)	308.8 ± 141.8	528.9 ± 159.8	< 0.01
Recovery of ^{59}Fe in femur (%)	0.4 ± 0.2	0.7 ± 0.2	< 0.01

Samples (N) Pregnant mice : spleens (18)
femurs (15)
Non-pregnant mice: spleens (5)
femurs (10)

DISCUSSION

Any study of the cellular kinetics of the thymus is complicated by the fact that the organ is essentially an open system. In the steady state, the rate of entry of stem cells from other lympho-myeloid tissue and the output of differentiated cells to peripheral organs also determine the cellularity of the thymus. These parameters have not been investigated in this study, which was concerned with that population of cells in the proliferative compartment.

The study of the cellularity of the thymus during pregnancy confirmed the earlier findings of other investigators that a pronounced wasting of the organ during pregnancy reduces it to between $\frac{1}{3}$ - $\frac{1}{4}$ of its normal size at parturition (Jolly and Lieure, 1930 and Maroni and De Sousa, 1973). The latter investigators claim that after syngeneic matings, the thymus was found to have increased in weight at all stages of gestation except at parturition. In contrast, it was found to regress progressively with increasing gestational age.

Splenic enlargement was observed after early gestation and was most pronounced at mid-gestation where it was increased by approx. $\frac{3}{4}$ of its normal size; thereafter it decreased in size but remained larger than normal at parturition. These observations are similar to those of Fowler and Nash (1968) who carried out a study of erythropoiesis in the spleens of pregnant mice. They showed that in the mouse, pregnancy constituted a haemopoietic stress. They observed that blood volume increased progressively

during pregnancy, resulting in a dilution anaemia. They found that the spleen doubled in weight by 12 days of gestation, with an increase of nucleated cells by x 3-4, thereafter it decreased in size and cellularity towards term. They report that splenic erythropoiesis increased rapidly to a peak at day 12 of gestation and declined thereafter, coincident with the decrease in spleen weight. They suggest that the alterations in the cellular profile of the spleen are a compensatory response to the pronounced anaemia during early pregnancy. The changes in spleen size observed in this study are not inconsistent with such a notion. Furthermore, the differential counts on nucleated spleen cells indicate a higher proportion of erythroid cells during pregnancy. Erythropoiesis in the spleens of pregnant mice was found to be greater to that in normal animals as determined by ^{59}Fe uptake. However, erythropoiesis in the bone marrow was found to be similar in both groups. This finding could be accounted for if erythropoiesis occurs preferentially in the spleen in response to haemopoietic stress. However, it is more likely that ^{59}Fe uptake in the marrow is less than what it should be because it is competitively taken up by the foetal liver. Thus the true magnitude of erythropoiesis in the bone marrow during pregnancy was probably not elucidated in this study. However, this contention cannot be substantiated as the foetal liver was not assayed for ^{59}Fe incorporation.

When the immune reactivity during pregnancy was assessed, by PHA responsiveness, modest differences in the reactivity between spleen and lymph node cells from pregnant animals were observed, lymph node cells responded to a slightly greater extent.

When the stimulation indices were compared between pregnant and normal animals, no clear differences were seen as the variability of the response between animals and experiments was large. However, the data from the differential counts do indicate that erythroid differentiation is more prevalent in the spleens of pregnant mice, the reduced lymphocytic count could account for any decreases in the PHA responsiveness during pregnancy. Ruppert and Richie (1977) cultured the nucleated cells from the spleens of pregnant mice at mid-gestation with PHA; they reported a reduced PHA responsiveness compared to normal animals, the stimulation index being as little as a $\frac{1}{6}$ of control animals. Following day 15, the response increased towards control levels, even exceeding the response of normal mice, at term. They also found that serum from post partum mice inhibited the blastogenic response to PHA. However, they failed to take into account the altered cellular composition of the spleen during pregnancy, which might have contributed significantly to any differences that they observed. The diminished responses they observed were most apparent at mid-gestation, when the spleen is at its largest and considered to be most active erythropoietically as suggested by Fowler and Nash (1968). The differential counts from the spleens of pregnant mice demonstrate that the numbers of lymphocytes may be reduced by $\frac{1}{3}$-$\frac{1}{4}$ of normal levels. However, this splenic lymphopenia does not reduce the PHA responsiveness by a corresponding amount. This effect was also seen in cell dose studies, cells cultured at 2×10^5/well often responded to a greater extent than cells at a higher concentration. Therefore, the PHA responsiveness is perhaps not the ideal parameter to experimentally

assess the immune status of animals in such conditions. The graft versus host capacity of splenic cells in irradiated recipients would probably give a more accurate and quantifiable indication of the degree of immunocompetence of the animals. In this investigation, lymph node cells generally responded to PHA more than spleen cells. This could be due to a more consistent and homogenous population of PHA responsive cells in the lymph nodes; whereas the splenic nucleated cells were a more heterogenous population, reflecting changes in the balance of haemopoietic function between animals, thus accounting for the variability of their PHA responsiveness. Since this study was carried out, Mattson et al (1979) have reported their observations after undertaking a similar study of splenic enlargement in pregnant mice. They also detect a slight increase in the degree of splenomegaly after allogeneic matings, proportional to the litter size which was noted in this investigation. They report a marked change in the structure of the splenic red pulp, with increased numbers of erythroblasts and plasma cells whereas T and B cell proportions were unaffected in mid-gestation, although a reduced Con A reactivity was detected.

The natural involution of the thymus during the period of experimental investigation was not considered to significantly affect the kinetic study. The period of investigation was generally between 2-6 weeks after the arrival of the animals, at a stage when the thymus regressed gradually.

The kinetic study confirmed primarily that cell proliferation was confined to the outer cortex of the thymus, particularly in the subcapsular region, as observed by other investigators (Kawamura,

1960 and Bryant, 1972). As immature lymphoid stem cells with the potential to divide are prevalent in the subcapsular region as noted by Kaplan (1961), it is not known whether cell proliferation in the subcapsular cortex is a consequence of inductive properties restricted to that region of the organ, or whether it is an intrinsic ability of the thymocytes themselves which is expended by the time they migrate to the medulla. Metcalf (1964) has estimated that greater than 97% of the lymphoid cells produced in the adult C_3H thymus were formed in the cortex. It cannot be determined whether mitotic cells arrested in metaphase in the various regions of the thymus entered into mitosis in those particular regions or whether they migrated there from regions nearer the capsule, already arrested in mitosis ie. whether proliferation is restricted to the subcapsular region. However, as mitotic cells continued to accumulate in the subcapsular cortex with time and as their numbers increased in other regions of the thymus without concomitant decreases in the subcapsular region, it is more likely that cell proliferation occurs throughout the cortex, but at different rates.

The cell production rates in normal and pregnant animals, calculated after linear regression analysis of the kinetic data are most informative, it was found to be a $\frac{1}{4}$ of the normal rate during pregnancy. In both conditions, there is a diminishing gradient of cell proliferation from the capsule to the medulla. This is in agreement with the findings of Kawamura (1960) in rats and Zaitoun et al (1979) in mice. The latter investigators found that prednisolone treatment had an inhibitory effect on the S and G_1 phases of the cell cycle, leading to a prolongation of the cell cycle

time. In addition, decreases in the thymocyte production rate and growth fraction were observed. Aherne et al (1980) found similar changes in the thymus of tumour-bearing animals. From this study, it was not possible to determine whether the cell cycle time was perturbed during pregnancy. However, if the changes in the cellularity of the thymus are caused by the increased corticosteroid levels, there is evidence to suggest that cell cycle parameters may be affected. Makman et al (1966) demonstrated that cortisol-treated rat thymocytes incorporated less radio-labelled precursors into proteins and nucleic acids in vitro than untreated controls. Frankfurt (1968) showed that adrenal corticosteroids inhibited cells from entering the S-phase. It is generally considered that the cell cycle time is relatively constant in mammalian tissues and does not change significantly in response to stress effects (A.C. Riches, personal communication). However, with an assumed mean cell cycle time of 9.0 h, the growth fraction in the proliferative regions of the cortex was decreased by an estimated 71%, which is greater than the 40% decrease caused by prednisolone treatment reported by Zaitoun et al (1979). These investigators also found that the cell production rate was reduced by about 60% and estimated a net reduction in the lymphocyte output of the thymus to be 25% of normal levels. By cautious extrapolation from their data, it is tentatively suggested that during pregnancy, the thymic lymphocyte output is further reduced.

It has been suggested that the numbers of stem cells alone determine the growth or involution of the organ, without significant

alterations in the cell cycle characteristics of the proliferating cells (Fabrikant and Foster, 1971). It was not possible from this study to determine whether the rate of entry of stem cells into the thymus was at all perturbed during pregnancy and can only be speculated upon. The changes in the cellularity of the spleen indicate that the organ responds in a compensatory manner to the haemopoietic stress caused by the parturient condition. At late gestation, the maternal bone marrow, spleen and foetal liver are haemopoietic and they could affect the normal rate of thymic lymphopoiesis by causing an imbalance of haemopoietic activity, particularly towards the erythroid lineage. The data from the ^{59}Fe uptake studies during late gestation support this contention.

There is evidence that during delayed hypersensitivity following contact sensitisation, there is an increased traffic of stem cells to the thymus from the bone marrow (Micklem et al, 1972). During the immune response following allogeneic skin grafting in rabbits, the draining lymph nodes were found to be significantly enlarged (Scothorne and McGregor, 1955). Maroni and De Sousa (1973) found the lumbar and renal lymph nodes draining the uterus were similarly enlarged, presumably as a consequence of antigenic challenge by the foetus. If it is assumed that both contact sensitisation and histoincompatible antigenicity elicit similar immunological responses, then it might be expected that more stem cells may be made available for thymic lymphopoiesis.

As mentioned previously, there are similarities between oncogenesis and embryogenesis. In addition to shared antigenicity,

the growth of the conceptus is more rapid than any tumour cell mass. There have been numerous investigations on the effects of steroid hormones, found in elevated levels during pregnancy, on the lymphoid tissue and immune reactivity, and studies on tumour immunity are prolific. This programme of enquiry has been an attempt to bestride two discrete themes in immunological research and the findings have further elucidated the cytokinetics of the thymus during a condition when immunological tolerance possibly prevails.

GENERAL DISCUSSION

In an early publication 'On the histogeny and involution of the thymus" in 1905, Hammar wrote ".... more and more interest has been shown in the said organ, observations are accumulating....'. Three quarters of a century later, further disclosures of its role in immune ontogeny continue to emerge and provide a fertile area for speculation.

A considerable part of this study has been directed towards attempting to isolate and enrich both the epithelial cells of the thymus and the precursors of T cells, intending to induce some degree of functional maturation in the latter by exposing them to the thymic microenvironment which would be approximated by their combination in vitro. This in retrospect, perhaps simplistic aim was found to pose enormous problems, compounded by the fact that both these cell types belong to heterogenous populations.

The morphology of thymic reticuloepithelial cells has been known for some time, the drawings of Hammar (1905) in Plates (20) and (21) correspond to the descriptions of what are believed to be thymic epithelium reported in more recent investigations. In histological sections, only the nuclei of these cells are apparent, having fine chromatin dots and a single nucleolus, the cytoplasm being inconspicuous as observed by Saint-Marie and Leblond (1964). Cells with similar morphological features were routinely observed in sections of explants of foetal thymus and were the predominant cells obtained after experimental treatments to enrich their proportion. The nuclei of the non-lymphoid adherent thymus cells cultured as monolayers also had a similar appearance ie. oval in shape with prominent nucleoli, but with discernible cytoplasm, as observed by other investigators (Waksal et al, 1975; Sato et al, 1976; and

Loor, 1979) and also in this study, both in situ and in cytocentrifuged preparations of trypsinised cells. It is therefore not surprising that these cells have been believed to be epithelial by most investigators. However, as mentioned before, recent investigations have elucidated the identity of these cells, the majority of which can be classified on the basis of their phagocytic and histochemical properties to be macrophages (Jordan et al, 1979a). Their findings have been confirmed in this study. The morphologically heterogenous cultured cells included fusiform and epithelioid shapes and resembled the cultured peripheral blood monocytes of other investigators (Zuckerman et al, 1979). The possibility that the monolayers contained a proportion of epithelial cells exists, as a few discrete areas of faintly PAS positive cells were seen, presumably epithelial. However, as PAS staining glycoproteins are a component of most cell surfaces, it may not necessarily mean they are epithelial secretory cells. It has been observed that PAS positive epithelial cells of the cortex can phagocytose pyknotic lymphocytes (Hoefsmit and Gerver, 1975) and epithelial cells of embryonic mouse thymus contain non-specific esterases (Smith, 1965). Berridge et al (1980) have similarly cultured the non-lymphoid adherent cells which they describe as the 'reticulum' cells of the thymus. They claim that autologous mouse serum supplements are an absolute requirement for the adherence and subsequent proliferation of these cells. This was not found to be so in this study, where proliferation of adherent cells was not impaired with foetal calf serum supplements. The morphological data they present does not differentiate their cultured cells from those in this study, the same degree of morphological heterogeneity being evident. They do not present data to discount

the possibility that their cultured cells may be macrophages and moreover admit to not detecting desmosomes or other junctional complexes, morphological features that were also not found in this study. In summary, it can be said that culture of non-lymphoid thymic tissue disaggregated by non-enzymic methods amplifies the endogenous macrophage population in preference to the epithelial component.

Attempts to detect Fc receptors for IgG on cultured thymic macrophages gave inconclusive results. They have been detected on human monocytic cells (LoBuglio, 1967 and Huber et al, 1969) and on murine monocytic cell lines (Koren et al, 1975), which have a similar morphology to thymic macrophages in culture. However, lack of definitive proof of their presence on these cells does not necessarily detract from their classification as macrophages. The expression of such receptors on macrophages may be a feature of their anatomical location and physiological function. Fc receptors on peripheral macrophages probably facilitate the cytophilic uptake of antigen-antibody complexes, whereas the blood-thymus barrier would effectively preclude such a function for thymic macrophages. The detection of Fc receptors on cultured peritoneal exudate cells lends support to this postulation. A similar study to detect Ia antigens would contribute further in characterising these cells and also reveal something of their functional significance. Rouse et al (1979) have stained thymic tissue with labelled antisera against MHC antigens and detect these in several different patterns on dendritic cells, Ia-bearing cells were present throughout the cortex. However, these patterns were not found to correspond with the known tissue distribution of thymic macrophages.

The differentiation inducing properties of such monolayer cells were found to be meagre, under the conditions of the assay system used to detect them ie. the reactivity of thymocytes to PHA. Although statistically significant differences (at $p < 0.05$) have been obtained, these were not considered to be biologically significant, as the responsiveness was compared to that of peripheral lymphocytes. It is interesting that a lesser but noticeable degree of stimulation was obtained after exposure to conditioned medium from monolayers of peritoneal exudate cells. This finding focuses attention on the question whether the properties of thymic macrophages are acquired as a consequence of their tissue location, which reiterates the indispensability of the thymus itself. The question also arises as to the nature of the factors and mechanisms responsible for the albeit modest increases in PHA reactivity of thymocytes exposed to the effects of thymic and peritoneal macrophages.

The role of the macrophage in immune phenomena has become increasingly apparent. Its function in antigen presentation, leading to helper effects (Pierce and Kapp, 1978 and Rosenthal et al, 1978) and the generation of suppressor mechanisms in its absence (Ishizaka and Adachi, 1976) are known. Furthermore, there has been an increasing body of evidence implicating its secretory products in the modulation of immune reactivity (reviewed by Waksman and Namba, 1976). Of these, lymphocyte activating factor (LAF) described by Gery and Waksman (1972) and mitogenic protein (MP) characterised by Farr et al (1977) stimulate the proliferation and enhance the mitogenic response of thymocytes. Their functions are not restricted to lymphopoietic activity as colony stimulating activity in granulopoiesis has been detected in a macrophage derived

factor (Chervenik et al, 1972). As mentioned previously, Unanue and co-workers have found that an active moiety, thymocyte differentiating factor (TDF) in the conditioned medium of peritoneal exudate macrophages is able to induce differentiation in immature thymocytes, the effect also being obtained when co-cultured with thymic macrophages (Beller and Unanue 1977, 1978 and Beller et al, 1978). These investigators used purified populations of immature thymocytes, whereas no attempt was made to enrich for these cells and whole thymocyte suspensions were used as targets in this study. Furthermore, they found that an incubation period of three days was needed to induce these maturational changes, whereas the cells were exposed to conditioned medium or monolayers for shorter periods in this study.

The T cell growth factors or interleukins have almost relegated the mitogenic role of lectins to second place. It is now believed that mitogens function by inducing the release of such factors by activated T cells, the actual process of mitosis being initiated by soluble signals, but requiring the presence of Ia-bearing accessory macrophages (Larsson and Coutinho, 1979 and Bödecker et al, 1980). The latter authors also found that interleukin 2 (IL-2) promoted cortisone resistant mature, but not peanut agglutinin positive immature thymocytes to proliferate. The earlier findings of Paetkau et al (1976) are relevant to these observations. They found that Con A induced the release of a lymphokine from thymus or spleen cells, which in the presence of a phytomitogen stimulated thymocyte proliferation. This Con A -induced co-stimulator enabled thymocytes to react to PHA as well as to Con A, the activity being dependent on the participation of macrophages bearing the Thy 1

It is apparent that the control of the immune response is mediated at least in part by T cells, macrophages and their respective secretory products. Chen and Di Sabato (1976) first described a class of T cell derived lymphokines, now called Interleukin 2 (IL-2) with a molec. wt. of 30-35000 daltons as having thymocyte stimulating activity. Its growth factor activity is demonstrated in its requirement in the maintenance of T cell lines in culture (Watson et al, 1979). It is now known that the elaboration of IL-2 requires the interaction of T cells as well as that of Ia-bearing macrophages (Andersson et al, 1979 and Shaw et al, 1980). In addition to antigen presentation, a primary role of the macrophage is the secretion of Interleukin 1 (IL-1), formerly known as lymphocyte activating factor (LAF), upon activation. IL-1 is thought to induce the release of IL-2 in the presence of antigen or mitogen (Smith et al, 1979). Furthermore, there is evidence that a soluble factor in supernatants of a macrophage cell line which exhibits IL-1 like activity can replace the need for macrophages to be present (Farrar et al, 1980 and Larsson et al, 1980). There is additional evidence that the interaction between T cells and macrophages enhances the secretion of IL-1 (Rosenstreich and Mizel, 1978).

In the light of these considerations, an alternative mechanism can be postulated to account for the modest increases in PHA reactivity of thymocytes observed in this study. The experimental system includes the necessary parameters for the production of IL-2 and the detection of its biological activity <u>in vitro</u>. It is

postulated that IL-2 is elaborated in the presence of both IL-1 and PHA by the cells that comprise the minor subpopulation of mature thymocytes which are known to have some of the properties of peripheral T cells. The same population could include cellular targets for IL-2 induced thymocyte stimulatory activity. It is possible that the cultured thymic macrophages could be in a state of continual activation due to the presence of xenogeneic serum supplements in the culture medium. They could therefore function as a source of IL-1, its effect being mediated either during initial co-culture with the thymocytes or during their incubation period in macrophage conditioned medium.

antigen. It is of interest to note that further work by Beller and Unanue (1979) suggest that two signals are necessary to initiate thymocyte proliferation by macrophage conditioned medium, the other being a non-mitogenic lectin pulse. In this study, an extraneous source of macrophages were not added to thymocytes during the actual period of culture and this could significantly impair their proliferative potential. It is possible to attribute the observed PHA responses to the mature cortisone resistant PHA reactive population of thymocytes, their low numbers accounting for the limited blastogenic response observed.

While most other investigators have attempted to induce maturation in immature lymphoid precursors in spleen and bone marrow (Waksal et al, 1975 and Sato et al, 1976), thymocytes were used as the target cells in this study. The rationale for this choice being the ease with which they could be obtained and the fact that they represented a population committed to the T-lineage of differentiation. However, as increasing evidence of the heterogeneity of thymocyte subpopulations (reviewed by Cantor and Weissman, 1976) and of multiple intra-thymic maturational pathways (Shortman and Jackson, 1974 and Scollay and Weissman, 1980) assembles, the use of thymocytes as putative targets would no doubt be complicated by these considerations.

It was decided to retract along the T-lineage pathway and attempt to enrich the population of pre-T cells in the bone marrow that were possibly in a determined state of differentiation. The findings of Komuro and Boyse (1973) indicated that such cells existed in the bone marrow. The bone marrow lymphocytes, believed to contain

the transitional cell population (Rosse, 1976) were not inducible by co-culture with thymus monolayer cells to express the Thy 1 antigen, bearing in mind that immunocytochemical techniques are limited by resolution and reagents. The small increases obtained with nude spleen cells suggests that this population rather than bone marrow might contain more inducible precursors, possibly the null cells. However, the Thy 1 antigen may not be restricted to thymus-derived cells. In the rat, it has been detected on immature haemopoietic cells (Hunt, 1979 and Goldschneider et al, 1978 and 1980) and on B cells (Crawford and Goldschneider, 1980). Thy 1 positive cells have been generated in cultures of nude spleen cells supplemented with supernatants from Con A stimulated normal spleen cells (Dennert and Hyman, 1980); this being further evidence for the thymic independence of Thy 1 expression, originally observed by Roelants (1976). Ritter and Morris (1980) have recently demonstrated the antigen on vascular basement membranes of lymphoid organs; therefore the induction of this membrane marker on T cell precursors may not necessarily represent a differentiative event that corresponds with maturational changes.

Bone marrow lymphocytes were not found to be inducible to PHA responsiveness upon prolonged incubation with the mitogen as claimed by Press and Rosse (1977a). It is suggested that the assay system was not sensitive enough to detect any probable increases in reactivity, rather than the lack of T-precursors in the marrow. Pro-thymocytes containing the enzyme terminal deoxyribonucleotidy transferase (Tdt), thought to be exclusive to immature T cells (Barton et al, 1976) have been enriched from bone marrow (Basch et al, 1978). It is possible that these cells require a prolonged maturational stay in the

environment of the marrow, where they are capable of generating T precursors under extrathymic influences. Recently, it has been shown that lymphoid cells could differentiate in vitro in long-term cultures of bone marrow in the system devised by Dexter et al (1977); furthermore colonies were derived that generated Thy 1-bearing cells after stimulation with PHA-induced conditioned medium from human leukocytes (Jones-Villeneuve and Phillips, 1980 and Jones-Villeneuve et al, 1980).

As the epithelial cells proved to be difficult to enrich in vitro, it was attempted to enrich their proportion in situ in an organ culture system which was considered would approximate the in vivo situation as far as possible. Lymphoid-depleted epithelial explants were obtained under conditions adverse to lymphoid growth, however attempts to re-introduce lymphoid precursors experimentally gave variable results. It is evident, bearing in mind the limitations of the system, that very immature haemopoietic cells were not able to repopulate these explants in vitro. Cell populations enriched for the CFU-S and bone marrow lymphocytes were not able to proliferate under these conditions. It is possible that they require a further maturational sojurn in other lymphomyeloid tissue or differentiate further in the bone marrow before they are able to seed the thymus. However, the introduction of CFU-S into the thymus may not be an entirely artificial situation, as these cells have been detected in foetal thymus (Barg et al, 1978). Therefore multipotentiality might still exist in lymphoid progenitors, committment being conferred only upon arrival in the appropriate haemopoietic enrivonment. More recently, Kraal et al (1980) have detected CFU-S in the regenerating thymuses of lethally

irradiated and reconstituted mice, and furthermore demonstrated a selective homing of labelled cells to the thymus upon a second transfer. The ability of lymphoid-depleted thymic explants to provide a suitable microenvironment for lymphopoiesis in vitro is demonstrated by the fact that syngeneic foetal thymocytes were able to proliferate within the organ. It might be argued that although the cultured explants appeared to be depleted of their lymphoid cells, a few surviving cells were amplified in subsequent culture conditions favourable for their growth. This possibility was discounted as the explants remained epithelial without prior reconstitution. Similar protocols have enabled lymphoid repopulation of epithelial explants by co-culture with foetal liver (R.K. Jordan, personal communication). From this study, it was found that fused composite embryonic organs facilitated experimental manipulation with ease by virtue of their larger size, whilst still being capable of rendering alymphoid and proving suitable for culture. Other investigators, for different reasons have depleted thyroid and pancreatic tissue of their passenger leukocytes in organ culture. Lacy et al (1979) have found isolated islets of Langerhans degenerated when cultured in an atmosphere predominantly composed of oxygen; however the toxic effects of high oxygen tension were overcome when large numbers of islets were fused together in culture (Lafferty, 1980). Thus although pancreated islets and foetal thymus may differ much in size, the same factors might determine the healthy survival of the tissue in organ culture.

While these experiments enabled the possibility of determining the thymus repopulating capacity of different cell populations, the

acquisition of functional and surface antigenic characteristics were not investigated. However, there is morphological evidence that cellular differentiation events do occur in the cultured explants in vitro following reconstitution. The regular appearance of numerous cellular structures containing a number of lymphoid cells indicates that some such process is underway. As mentioned before, these are probably the giant "nurse cells" described by Wekerle and Ketelsen (1980) and Wekerle et al (1980). They are believed to be epithelial in character and express MHC coded antigens but lack the lymphocyte differentiation markers; the antigenic make-up being identical to that of thymic macrophages, though they were not found to be phagocytic for non-cellular matter. If these nurse cells are a hybrid with the morphological features of epithelium and functional attributes of macrophages, it might be worthwhile to assay these cells for secretion of thymic factors and esterase activity. Macrophages are known to possess an extensive complement of enzymes (Unanue et al, 1976). It is tempting to speculate that these cells function as specialised macrophages which selectively endocytose only lymphoid cells, recognition being mediated by the array of antigens displayed on their surfaces. Exposure of immature lymphoid cells to the cytoplasmic enzymic milieu might unmask differentiation antigens or trigger certain maturational events prior to their subsequent release. It is of relevance to these speculations that proteolytic enzymes have been shown to potentiate the blastogenic response of rat thymocytes to Con A (Ulrich et al, 1979). The phenomenon of lymphocytes within the cytoplasm of other cells (emperipolesis) has been known for some time, especially within epithelial cells. Andrew and Sosa (1947)

reported their presence within mouse intestinal epithelial cells. It was once considered that almost all epithelial cells could be phagocytic on occasion (Trowell, 1958); therefore it is possible that these observations have no immunological relevance. However, the fact that they occur in the thymus and express MHC antigens discounts that possibility.

Cellular interactions between lymphocytes and macrophages have been recognised for some time. Schonenberg et al (1964) observed the association of these cell types and presented ultrastructural evidence for the transfer of material from macrophages to lymphocytes. J.A. Sharp (1966 and 1971) observed large motile cells emerge from explants of rabbit thymus in culture, forming close associations with a number of trailing lymphocytes. Of relevance to these cellular interactions is the now recognised phenomenon of MHC restriction (reviewed by Zinkernagel and Doherty, 1979). Thymus lymphocytes are believed to encounter self-antigens on epithelial cells within the thymus. Subsequent cell mediated effector functions in the periphery requires recognition of foreign antigens in association with MHC antigens of the phenotype first encountered whilst being "processed" in the thymus. It has been reported that upon co-culture with thymic epithelial monolayers, H.2 restriction could be induced in pre-thymic cells of the bone marrow, of the same specificity as the epithelium; a post thymic population was found to be refractory to such induction in vitro (Gorczynski et al, 1979).

The kinetic study of thymus lymphocytes firstly demonstrated that the greatest proliferative activity was in the subcapsular region, an area rich in macrophages. The earlier observations of Metcalf

and Ishidate (1961) report that a higher frequency of mitotic lymphocytes were found in close association with PAS positive cells of varying size between 7-26 microns. Their cytoplasm containing phagocytosed pyknotic lymphoid cells and surrounded by a cluster of lymphocytes, some being found to invaginate the cytoplasm of the larger cells. Furthermore, these cells were found most frequently in the subcapsular region. It is possible that the large motile cell (J.A. Sharp, 1966), the nurse cell (Wekerle and Ketelsen, 1980) the ones observed in this study are the same or have very similar functions which are not yet fully understood. If these cells are in fact macrophages or functionally equivalent to them, their possible proliferation inducing properties would not be surprising. Therefore, the mitogenic effects of the secretory products of thymic macrophages may be an in vitro correlate of their properties in vivo.

The diminished thymocyte proliferation rates seen during pregnancy can be accounted for by several factors, as discussed previously. These changes were seen in CDI animals, an outbred strain; therefore a comparative study with syngeneic matings in an inbred strain might further elucidate the contribution of hormonal and immunoregulatory causative factors. The findings from the kinetic study should be considered in conjunction with the long-held belief that the immune vigour is impaired during pregnancy, and the observation that T and B cell levels undergo an inversion during the first trimester in human pregnancies (Strelkauskas et al, 1975). If the human thymus continues to regress throughout pregnancy as in the mouse, it does not explain the reversion of T and B cells to normal proportions towards parturition. A similar

quantitative study of T and B cell populations throughout pregnancy in the mouse may therefore prove worthwhile, especially if correlated with cell production rates in the thymus.

To conclude with perhaps a frivolous analogy, the cortical thymocyte like the lemming has a high incidence of premature death, the consequence of a protective mechanism against autoreactive clones (Burnet, 1962). During pregnancy, additional unresolved factors generate fewer numbers of potentially reactive T cells, which ensures the survival of the embryo against what can be considered the immunological hazards to life before birth.

BIBLIOGRAPHY

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. Anat. Rec. 94 : 239-247

Abramson, S., Miller, R.G. and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med. 145 : 1567-1579

Andersson, J., Gronvik, K.O., Larsson, E-L. and Coutinho, A. (1979). Studies on T lymphocyte activation 1. Requirements for the mitogen dependent production of T cell growth factors. Eur. J. Immunol. 9 : 581-587

Andreasen, E. and Christensen, S. (1949). The rate of mitotic activity in the lymphoid organs of the rat. Anat. Rec. 103 : 401-411

Andrew, W. and Sosa, J.M. (1947). Mitotic division and degeneration of lymphocytes within cells of intestinal epithelium in young and in adult white mice. Anat. Rec. 97 : 63-84

Asanuma, Y., Goldstein, A.L. and White, A.(1970). Reduction in the incidence of wasting disease in neonatally thymectomised CBA/W mice by the injection of thymosin. Endocrinology 86 : 600-610

Astaldi, G., Astaldi, A., Groenewoud, M., Wijermans, P. and Eijswoogel, V.P. (1977). Effect of human serum factor on hydrocortisone treated thymocytes. Eur. J. Immunol. 7 : 836-840

Auerbach, R. (1960). Morphogenetic interactions in the development of the mouse thymus gland. Dev. Biol. 2: 271-284

Auerbach, R.(1961). Experimental analysis of the origin of cell types in the development of the mouse thymus. Dev. Biol. 3 : 336-354

Bach, J.F., Bach, M.A., Charreire, J., Dardenne, M., Fournier, C., Papiernik, M. and Pleau, J-M. (1975). The circulating thymic factor (TF). Biochemistry, physiology, biological activities and clinical applications. A summary. in, The biological activity of thymic hormones. (Ed. Van Bekkum, D.W.) Kooyker Scientific Publications, Rotterdam p. 145-158

Bach, J.F. and Carnaud, C. (1976). Thymic factors. Progr. Allergy 21 : 342-408

Bach, J.F. and Dardenne, M. (1973). Studies of thymus products. II. Demonstration and characterisation of a circulating thymic hormone. Immunology 25 : 353-336

Bach, J.F., Muller J-Y. and Dardenne, M., Goldstein, A.L., Guha, A. and White, A. (1971). Appearance of T cell markers in bone marrow rosette-forming cells after incubation with thymosin, a thymic hormone. P.N.A.S. 68 : 2734-2738

Bach, J.F., Dardenne, M., Papiernik, M. and Barois, A. (1972). Evidence of a serum factor secreted by the human thymus. Lancet II : 1056-1058

Ball, W.D. and Auerbach, R. (1960). In vitro formation of lymphocytes from embryonic thymus. Exp. Cell. Res. 20 : 245-247

Barg, M., Mandel, T.E. and Johnson, G.R. (1978). Haemopoietic stem cells in the foetal mouse thymus. Aust. J. Exp. Biol. and Med. Sci. 56 : 195-200

Barton, R., Goldschneider, I. and Bollum, F.J. The distribution of terminal deoxyribonucleotidyl transferase (Tdt) among subsets of thymocytes in the rat. J. Immunol. 116 : 462-468

Basch, R.S. and Goldstein, G. (1974). Induction of T cell differentiation in vitro by thymin (thymopoietin), a purified polypeptide hormone of the thymus. P.N.A.S. 71 : 1474-1478

Basch, R.S. and Goldstein, G. (1975). Thymopoietin-induced acquisition of responsiveness to T cell mitogens. Cell. Immunol. 20 : 218-228

Basch, R.S., Kadish, J.L. and Goldstein, G. (1978). Haemopoietic thymocyte precursors IV. Enrichment of the precursors and evidence for heterogeneity. J. Exp. Med. 147 : 1843-1848

Beller, D.I. and Unanue, E.R. (1977). Thymic maturation in vitro by a secretory product from macrophages. J. Immunol. 118 : 1780-1787

Beller, D.I. and Unanue, E.R. (1978). Thymic macrophages modulate one stage of T cell differentiation in vitro. J. Immunol. 121 : 1861-1864

Beller, D.I. and Unanue, E.R. (1979). Evidence that thymocytes require at least two distinct signals to proliferate. J. Immunol. 123 : 2890-2893

Beller, D.I., Farr, A.G. and Unanue, E.R. (1978). Regulation of lymphocyte proliferation and differentiation by macrophages. Fed. Proc. 37 : 91-96

Berridge, M.V., Narang, J.C., Loverdale, P.T. and GoTub, E.S. (1980). The culture of mouse thymic reticulum cells: The effect of homologous serum on the establishment of primary cultures. J. Immunol. 124 : 2738-2746

Billingham, R.E., Krohn, P.L. and Medawar, P.B. (1951). Effects of locally applied cortisone acetate on survival of skin homografts in rabbits. B.M.J. 2 : 1049-1051

Blomgren, H. and Andersson, B. (1969). Evidence for a small pool of immuno-competent cells in the mouse thymus. B.M.J. 2 : 1049-1051

Blomgren, H. and Andersson, B. (1971). Characteristics of the immunocompetent cells in the mouse thymus: Cell population changes during cortisone-induced atrophy and subsequent regeneration. Cell. Immunol. 1 : 545-560

Blomgren, H. and Svedmyr, E. (1971). Evidence for thymic dependence of PHA reactive cells in spleen and lymph nodes and independence in bone marrow. J. Immunol. 106 : 834-841

Bödecker, B.G.D. (1980). Mitogenic effects of partially purified interleukin 2 on thymocyte subpopulations and spleen T cells of the mouse. Eur. J. Immunol. 10 : 702-709

Boyse, E.A. and Old, L.J. (1969). Some aspects of normal and abnormal cell surface genetics. Ann. Rev. Genet. 3 : 269-290

Brown, M.B. (1953). Effect of intravenously injected bone marrow cell suspensions on thymic regeneration in irradiated C57 black mice. Science 117 : 693-695

Bryant, B.J. (1972). Renewal and fate in the mammalian thymus: mechanisms and influences of thymocytokinetics. Eur. J. Immunol. 2 : 38-45

Bullough, W.S. and Laurence, E.B. (1960). The control of epidermal mitotic activity in the mouse. Proc. Roy. Soc.(London) Ser. B. 151 : 517-536

Burleson, R. and Levey, R.H. (1971). Demonstration of thymic function in vitro. Transplant. Proc. 111 : 918-922

Burleson, R. and Levey, R.H. (1972). Studies on the isolation of lymphocytes active in cell mediated immune responses. I. Demonstration of an active population of thymus derived cells in mouse bone marrow. Cell. Immunol. 4 : 305-315

Burnet, F.M. (1962). Role of the thymus and related organs in immunity. B.M.J. 2 : 807-811

Cantor, H. and Boyse, E.A. (1977). Regulation of the immune response by T cell subclasses. in, Contemporary topics in immunology. Vol. 7 : T Cells (Ed. Stutman, O.) Plenum, N.Y., pp. 47-67

Cantor, H. and Weissman, I.L. (1976). Development and function of subpopulations of thymocytes and T lymphocytes. Progr. Allergy 20 : 1-64

Carnaud, C., Ilfeld, D., Brook, I. and Trainin, N. (1973). Increased reactivity of mouse spleen cells sensitised in vitro against syngeneic tumour cells in the presence of a thymic humoral factor. J. Exp. Med. 138 : 1521-1532

Castro, J.E. (1974). Orchidectomy and the immune response I. Effect of orchidectomy on lymphoid tissue of mice. Proc. Roy. Soc. (London) Ser. B. 185 : 424-436

Chakravarty, A., Kubai, L., Sidky, Y. and Auerbach, R. (1975). Ontogeny of thymus cell function. Ann. N.Y. Acad. Sci. 249 : 34-42

Chen, D.M. and Di Sabato, G. (1976). Further studies of the thymocyte stimulating factor. Cell. Immunol. 22 : 211-224

Chen, J.M. (1954). The cultivation in fluid medium of organised liver, pancreas and other tissues of foetal rats. Exp. Cell. Res. 7 : 518-529

Chervenick, P.A. and Lo Buglio (1972). Human blood monocytes: stimulators of granulocyte and mononuclear colony formation in vitro. Science 178 : 164-166

Clark, S.L. (1963). The thymus in mice of strain 129/J studied with the electron microscope. Am. J. Anat. 112 : 1-33

Clark, S.L. (1968). Incorporation of sulphate by the mouse thymus: its relation to secretion by medullary epithelial cells and to thymic lymphopoiesis. J. Exp. Med. 128 : 927-957

Clark, S.L. (1973). The intrathymic environment. in, Contemporary topics in Immunobiology, Vol. 2 : Thymus dependency (Eds. Davies, A.J.S. and Carter, R.L.), Plenum N.Y.,pp. 77-99

Cohen, J.J. and Fairchild, S. (1976). T-lymphocyte precursors I. Synergy between precursor and mature T lymphocytes in the response to Con A. J. Exp. Med. 144 : 456-466

Cohen, J.J. and Patterson, C.K. (1975). Induction of theta positive lymphocytes and lymphoblasts in mouse bone marrow by mitogens. J. Immunol. 114 : 374-376

Colley, D.G., Wu, A.Y.S. and Waksman, B.H. (1970). Cellular differentiation in the thymus III. Surface properties of rat thymus and lymph node cells separated on density gradients. J. Exp. Med. 132 : 1107-1121

Cordier, A.C. (1974). Ultrastructure of the thymus in nude mice. J. Ultrastruct. Res. 47 : 26-40

Craddock, C.G., Nakal, G.S., Fukutu, H. and Vanslanger, L.M. (1964). Proliferative activity of the lymphatic tissues of rats with tritum labelled thymidine. J. Exp. Med. 120 : 389-412

Crawford, J.M. and Goldschneider, I. (1980). Thy 1 antigen and B lymphocyte differentiation in the rat. J. Immunol. 124 : 969-976

Csaba, G., Toro, I., Aes, T. and Kiss, F.I. (1960). The behaviour of the thymus in conditions associated with tissue proliferation. Acta Morphologica Hungarica 9 : 187-196

Dalmasso, A.P., Martinez, C., Sjodin, K. and Good, R.A. (1963). Studies on the role of the thymus in immunobiology: Reconstitution of immunologic capacity in mice thymectomised at birth. J. Exp. Med. 118 : 1089-1109

Dardenne, M. and Bach, J.F. (1973). Studies on thymus products I. Modification of rosette forming cells by thymic extracts. Determination of the target RFC subpopulation. Immunology, 25 : 343-352

Dennert, G. and Hyman, R. (1980). Functional Thy 1 positive cells in cultures of spleen cells from nu/nu mice. Eur. J. Immunol. 10 : 583-590

De Sousa, M.A.B., Parrott, D.M.V. and Pantelouris, E.M. (1969). The lymphoid tissues in mice with congenital aplasia of the thymus. Clin. Exp. Immunol. 4 : 637-644

Dexter, T.M., Allen, T.D. and Lajtha, L.G. (1977). Conditions controlling the proliferation of haemopoietic cells in vitro. J. Cell. Physiol. 91 : 335-344

Dicke, K.A., Hooft, J.I.M. and Van Bekkum, D.W. (1968). The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures III. Mouse spleen cell fractionation on a discontinuous albumin gradient. Transplantation 6 : 562-573

Dougherty, T.F. (1964). Hormonal control of lymphatic structure and function. Ann. N.Y. Acad. Sci. 113 : 689-694

Dröege, W. and Zucker, R. (1975). Lymphocyte subpopulations in the thymus. Transplant. Rev. 25 : 3-25

El-Arini, M.O. and Osoba, D. (1973). Differentiation of thymus-derived cells from precursors in mouse bone marrow. J. Exp. Med. 137 : 821-837

Elliott, E.V. (1973). A persistent lymphoid cell population in the thymus. Nature 242 : 150-151

Emmart, E.W. (1936). A study of the histogenesis of the thymus in vitro. Anat. Rec. 66 : 59-73

Ernstrom, U., Gyllensten, L. and Larsson, B. (1965). Venous output of lymphocytes from the thymus. Nature 207 : 540-541

Fabrikant, J.I. and Foster, B.R. (1971). Cell population kinetics in lymphocytes in mouse and rat thymuses. Johns Hopkins Med. J. 130 : 208-215

Farr, A G., Dorf, M.E. and Unanue, E. (1977). Secretion of mediators following T lymphocyte-macrophage interaction is regulated by the major histocompatibility complex. P.N.A.S. 74 : 3542-3546

Farrar, J.J., Mizel, S.B., Fuller-Bonar, J., Hilfiker, M.L. and Farrar, W.L. (1980). Lipopolysaccharide-mediated adjuvenicity effect of lipopolysaccharide on the production of T cell growth factor (Interleukin 2) in, Microbiology 1980. Ed. Schlesinger,D. pp. 36-39

Fathman, C.G., Small, M., Herzenberg, L.A. and Weissman, I.L. (1975). Thymus cell maturation II. Differentiation of three mature subclasses in vivo. Cell. Immunol. 15 : 109-128

Fialkow, P.J. (1977). Clonal origin and stem cell evolution of human tumours. in, Genetics of human cancer. Eds, Mulvihill, J.J., Miller, R.W. and Fraumeni, J.F., Raven Press, N.Y., pp. 439-450

Fialkow, P.J., Denman, A.M., Singer, J., Jacobsen, R.J. and Lowenthal, M.N. (1980. Human myeloproliferative disorders: clonal origin in pluripotent stem cells. in, Differentiation of normal and neoplastic haemopoietic cells. Eds, Clarkson, B. Marks, P.A. and Till, J.E., Coldspring Harbor Laboratory, N.Y., pp. 131-144

Ford, C.E., Micklem, H.S., Evans, E.P., Gray, J.G. and Ogden, D.A. (1966). The inflow of bone marrow cells to the thymus: studies with part-body irradiated mice injected with chromosome-marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci., 129 : 283-296

Fowler, J.H. and Nash, D.J. (1968). Erythropoiesis in the spleen and bone marrow of the pregnant mouse. Dev. Biol. 18 : 331-353

Frankfurt, O.S. (1968). Effect of hydrocortisone, adrenaline and actinomycin D on transition of cells to the DNA synthesis phase. Exp. Cell. Res. 52 : 222-232

Garaci, E., Pecci, G., Rinaldi-Garaci, C., Del Gobbo, V. and Tonietti, G. (1978). Ultrastructural and functional changes of the mouse thymus following treatment with an antiserum specific for thymic epithelial cells. Clin. Immunol. Immunopath. 11 : 157-165

Gengozian, N., Urso, I.S., Congdon, C.C., Conger, A.D. and Makinodan, T. (1957). Thymus specificity in lethally irradiated mice treated with rat bone marrow. Proc. Soc. Exp. Biol. and Med. 96 : 714-720

Gershwin, M.E., Ahmed, A., Steinberg, A.D., Thurman, G.B. and Goldstein, A.L. (1974). Correction of T cell function by thymosin in New Zealand mice. J. Immunol. 113 : 1068-1071

Gery, I. and Waksman, B.H. (1972). Potentiation of T-lymphocyte response to mitogens II. The cellular source of potentiating mediators. J. Exp. Med. 136 : 143-155

Goldschneider, I., Gordon, L.K. and Morris, R.J. (1978). Demonstration of Thy 1 antigen on pluripotent haemopoietic stem cells in the rat. J. Exp. Med. 148 : 1351-1366

Goldschneider, I., Metcalf, D., Battye, F and Mandel, T. (1980). Analysis of rat haemopoietic cells on the FACS I. Isolation of pluripotent haemopoietic stem cells and granulocyte-macrophage progenitor cells. J. Exp. Med. 152 : 419-437

Goldstein, A.L., Thurman, G.B., Cohen, G.H. and Hooper, J.A. (1975). Thymosin : Chemistry, biology and clinical application. in, The biological activity of thymic hormones (Ed. Van Bekkum) Kooyker Scientific Publications, Rotterdam, pp. 173-197

Goldstein, A.L., Slater, F. and White, A. (1966). Preparation, assay and partial purification of a thymic lymphocytopoietic factor (Thymosin). P.N.A.S. 56 : 1010-1017

Goldstein, A.L., Asanuma, Y., Battisto, J.R., Hardy, M.A. and White, A. (1970). Influence of thymosin on cell mediated and humoral immune responses in normal and in immunologically deficient mice. J. Immunol. 104 : 359-366

Goldstein, A.L., Guha, A., Katz, M.M., Hardy, M.A. and White, A. (1972). Purification and biological activity of thymosin, a hormone of the thymus gland. P.N.A.S., 69 : 1800-1803

Goldstein, G. (1974). Isolation of bovine thymin: a polypeptide hormone of the thymus. Nature 247 : 11-14

Gorczynski, R.M. and MacRae, S. (1977a). Differentiation of functionally active mouse T lymphocytes from functionally inactive bone marrow precursors I. Kinetics of recovery of T cell function in lethally irradiated, bone marrow reconstituted Tx and non-Tx mice. Immunology 33 : 697-712

Gorczynski, R.M. and MacRae, S. (1979). Differentiation of functionally active mouse lymphocytes from functionally inactive bone marrow precursors III. Induction of T cell activities by growth of bone marrow on feeder layers prepared from mouse thymocytes. Immunology 38 : 1-12

Gorczynski, R.M., Khomasurya, B. and MacRae, S. (1979). MHC restriction of murine T lymphocyte reactivity analysed by growth of bone marrow cells on thymic epithelial monolayers. Immunology 39 : 835-846

Gregoire, Ch. (1947). Factors involved in maintaining involution of the thymus during suckling. J. Endocr. 5 : 68-87

Ham, A. (1977). Ham's Histology. 7th edition, J.B. Lippincott Co., Philadelphia, pp. 326-334.

Hammar, J.A. (1905). On the histogeny and involution of the thymus. Anat. Anz. 27 : 23-43

Harper, C.M. and Sharp, J.G. (1980). Re-evaluation of the morphological and functional characteristics of thymic non-lymphoid cells in vitro. In press

Harris, J.E. and Ford, C.E. (1963). The role of the thymus: Migration of cells from thymic grafts to lymph nodes in mice. Lancet 1 : 389-390

Harris, J.E. and Ford, C.E. (1964). Cellular traffic of the thymus: Experiments with chromosomal markers. Nature 201 : 884-885

Harris, J.E., Ford, C.E., Barnes, D.W.H. and Evans, E.P. (1964). Evidence from parabiosis for an afferent stream of cells. Nature 201 : 886-887

Hau, T. (1975). HCG - its inhibitory effect on cell mediated immunity in vivo and in vitro. Immunology 29 : 509-515

Hellström, K.E., Hellstrom, I. and Brawn, J. (1969). Abrogation of cellular immunity to antigenically foreign mouse embryonic cells by a serum factor. Nature 224 : 914-915

Heslop, R.W., Krohn, P.L. and Sparrow, E.M. (1954). The effect of pregnancy on the survival of skin homografts in rabbits. J. Endocr. 10 : 325-332

His (1885). as quoted by Metcalf and Moore, 1971.

Hoefsmit, E.C.M. and Gerver, J.A.M. (1975). Epithelial cells and macrophages in the normal thymus. in, The biological activity of thymic hormones (Ed. D.W. Van Bekkum) Kooyker Scientific Publications, Rotterdam, pp. 63-68

Hong, R., Schulte-Wisserman, H., Jarrett-Toth, E. and Horowitz, S. (1979). Transplantation of cultured thymic fragments II. Results in nude mice. J. Exp. Med. 149 : 398-415

Huber, B,. Cantor, H., Shen, F.W. and Boyse, E.A. (1976). Independent differentiative pathways of Ly 1 and Ly 23 subclasses of T cells. J. Exp. Med. 144 : 1128-1133

Huber, H., Douglas, S.D. and Fudenberg, H.H. (1969). The IgG receptor : an immunological marker for the characterisation of mononuclear cells. Immunology 17 : 7-21

Hunt, S.V. (1979). The presence of Thy 1 on the surface of rat lymphoid stem cells and colony-forming units. Eur. J. Immunol. 9 : 853-859

Ishizaka, K. and Adachi, T. (1976). Generation of helper cells and suppressor cells in vitro for the IgE and IgG antibody responses. J. Immunol. 117 : 40-47

Joel, D.D., Chanana, A.D., Cottier, H., Cronkite, E.P. and Laissue, J.A. (1977). Fate of thymocytes: Studies with ^{125}Iodo-deoxyuridine and ^{3}H-Thymidine in mice. Cell. Tissue Kinet. 10 : 57-69

Jolly, J. and Lieure, C. (1930). Influence de la gestation suv le thymus. Compt. Rend. Soc. de Biol. 114 : 451-452

Jones-Villeneuve, E.V. and Phillips, R.A. (1980). Potentials for lymphoid differentiation by cells from long-term cultures of bone marrow. Exp. Haemat. 8 : 65-76

Jones-Villeneuve, E.V., Rusthaven, J.J., Miller, R.G. and Phillips, R.A. (1980). Differentiation of Thy-1-bearing cells from progenitors in long-term bone marrow cultures. Eur. J. Immunol. 10 : 597-603

Jordan, R.K. and Crouse, D.A. (1979a). Studies on the thymic microenvironment: Morphologic and functional characterisation of thymic non lymphoid cells in tissue culture. J. Reticuloendothel. Soc. 26 : 385-399

Jordan, R.K. and Crouse, D.A (1979b). Non-lymphoid cells and intrathymic T cell differentiation. in, Symp. Dev. Biol (in press)

Jordan, R.K., Crouse, D.A., Harper, C.M., Watkins, E.B. and Sharp, J.G. (1979a). The thymic microenvironment. Exp. Haemat. Today pp. 139-153

Jordan, R.K., Crouse, D.A. and Owen, J.J.T. (1979b). Studies on the thymic microenvironment: Non lymphoid cells responsible for transferring the microenvironment.
J. Reticuloendothel. Soc. 26 : 373-383

Juhlin, R., Tufveson, G. and Alm, G.V. (1977). Role of the thymus in generation of lymphocyte functions II. Serum-mediated inhibition of development of mitogen reactive lymphocytes in embryonic mouse thymus organ culture.
Cell. Immunol. 32 : 193-202

Kaplan, H.S. (1950). Influence of thymectomy, splenectomy and gonadectomy on the incidence of radiation induced lymphoid tumours in strain C57B1 mice. J.Nat. Cancer Inst. 11 : 83-90

Kaplan, H.S. (1961). The role of cell differentiation as a determinant of susceptibility to virus carcinogenesis. Cancer Res. 21 : 981-983

Kaplan, H.S., Nagareda, C.S. and Brown, M.B. (1954). Endocrine factors and radiation-induced lymphoid tumours of mice.
Rec. Progr. Hormone Res. 10 : 293-338

Kawamura, T. (1960). Variations in mitotic activity in different regions of lymphoid organs I. Observations of the thymo-lymphatic organs of young mature albino rats.
Folia Anat. Japan 35 : 345-365

Kindred, B. and Loor, F. (1974). Activity of host-derived T cells which differentiate in nude mice grafted with co-isogeneic or allogeneic thymus. J. Exp. Med. 139 : 1215-1227

Kindred, J.E. (1940). A quantitative study of the haemopoietic organs of young albino rats. Am. J. Anat. 67 : 99-149

Klein, J., Goldstein, A.L. and White, A. (1965). Enhancement of in vivo incorporation of labelled precursors into DNA and total protein of mouse lymph nodes after administration of thymic extracts. P.N.A.S. 53 : 812-817

Kollicker, A. (1879). as quoted by Metcalf and Moore (1971)

Komuro, K. and Boyse, E.A. (1973). Induction of T lymphocytes from precursor cells in vitro by a product of the thymus. J. Exp. Med. 138 : 479-482

Komuro, K., Goldstein, G. and Boyse, E.A. (1975). Thymus repopulating capacity of cells than can be induced to differentiate in vitro. J. Immunol. 115 : 195-198

Konda, S., Nakao, Y. and Smith, R.T. (1972). Immunologic properties of mouse thymus cells: Identification of T cell functions within a minor, low density subpopulation. J. Exp. Med. 136 : 1461-1477

Koren, H.S., Handwergen, B.S. and Wunderlich, J.R. (1975). Identification of macrophage-like characteristics in a cultured murine tumour line. J. Immunol. 114 : 894-897

Kotani, M., Seiki, K., Yamashita, A. and Horii, I. (1966). Lymphatic drainage of thymocytes to the circulation in the guinea pig. Blood 27 : 511-520

Kraal, G., Geldof, A.A. and Boden, D. (1980. T cell differentiation in lethally irradiated and reconstituted mice. Cell. Immunol. 49 : 110-119

Kruisbeek, A., Krose, T.C.J.M. and Ziglstra, J.L. (1977). Increase in T cell mitogen responsiveness in rat thymocytes by thymic epithelial culture supernatants. Eur.J.Immunol. 7 : 375-381

Kruisbeek, A., Astaldi, G.C.B., Blankwater, M.J., Zilgstra, J.L. and Astaldi, A. (1978). The in vitro effect of a thymic epithelial culture supernatant on MLR and intracellular cAMP levels of thymocytes and on antibody production to SRBC by nu/nu spleen cells. Cell. Immunol. 35 : 134-147

Lacy, P.E., Davie, J.M. and Finke, E.H. (1979). Prolongation of islet allograft survival following in vitro culture (24°C) and a single injection of ALS. Science 204 : 312-313

Lafferty, K.J. (1980). Immunogenicity of foreign tissues. Transplantation 29 : 179-181

Lafferty, K.J., Cooley, M.A., Woolnough, J. and Walker, K.Z. (1975). Thyrmoid allograft immunogenicity is reduced after a period in organ culture. Science 188 : 259-261

Laissue, J.A., Chanana, A.D., Cottier, H., Cronkite, E.P. and Joel, D.D. (1976). The fate of thymic radioactivity after local labelling with ^{125}-Iodo-deoxyuridine. Blood 47 : 21-30

Larsson, E-L. and Coutinho, A. (1979). The role of mitogenic lectins in T cell-triggering. Nature 280 : 239-241

Larsson, E-L., Coutinho, A. and Martinez, C. (1980). A suggested mechanism for T lymphocyte activation: implication on the acquisition of functional reactivities. Immunological Rev. 51 : 61-91

Law, L.W. (1964). Humoral thymic factor in mice: Further evidence. Science 143 : 1049-1051

Leckband, E. and Boyse, E.A. (1971). Immunocompetent cells among mouse thymocytes : a minor population. Science 172 : 1258-1260

Le Douarin, N.M. (1980). The early events in thymus ontogeny studied in embryonic chimaeras. To be published in, The Proceedings of the Anatomical Society of Gt. Britain and Ireland, 1980

Le Douarin, N.M. and Jotereau, F.V. (1975). Tracing of cells of the avian thymus through embryonic life in interspecific chimaeras. J. Exp. Med. 142 : 17-40

Levey, R.H. and Burleson, R. (1972). Studies on the isolation of lymphocytes active in cell-mediated immune responses II. Identification and recovery of an immunocompetent subpopulation of mouse thymocytes. Cell. Immunol. 4 : 316-332

Levey, R.H., Trainin, N., Law, L.W., Black, P.H. and Rowe, W.P. (1963a). Lymphocytic choriomeningitis infection in neonatally thymectomised mice bearing diffusion chambers containing thymus. Science 142 : 483-485

Levey, R.H., Trainin, N. and Law, L.W. (1963b). Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomised mice. J. Nat. Cancer Inst. 31 : 199-218

Lis, H. and Sharon, N. (1973). The biochemistry of plant lectins (Phytohaemagglutinins). Ann. Rev. Biochem. 42 : 541-574

LoBuglio, A.F. (1967). Red cells coated with Immunoglobulin G: Binding and sphering by mononuclear cells in man. Science 158 : 1582-1585

Lonai, P., Mogilner, B., Rotter, V. and Trainin, N. (1973). Studies on the effect of a thymic humoral factor on differentiation of thymus derived lymphocytes. Eur. J. Immunol. 3 : 21-26

Loor, F. (1979). Mouse thymus reticulo-epithelial (RE) cells in vitro : Isolation, cultivation and preliminary characterisation. Immunology 37 : 157-177

Loor, F. and Hägg, L.B. (1977). The restoration of the T-lymphoid system of nude mice : Lower efficiency of non-lymphoid, epithelial thymus grafts. Cell. Immunol. 29 : 200-209

Loor, F. and Kindred, B. (1973). Differentiation of T cell precursors in nude mice demonstrated by immunofluorescence of T cell membrane markers. J. Exp. Med. 138 : 1044-1055

Loor, F., Amstutz, H., Hagg, L-B., Mayer, K.S. and Roelants, G.S. (1976). T lineage lymphocytes in nude mice born from homozygous nu/nu parents. Eur. J. Immunol. 6 : 663-665

Low, T.L.K. and Goldstein, A.L. (1979). Thymosin and other thymic hormones. Springer Semin. Immunopathol. 2 : 169-186

Makman, M.H., Dvorkin, B. and White, A. (1966). Alterations in protein and nucleic acid metabolism of thymocytes produced by adrenal steroids in vitro. J. Biol. Chem. 241 : 1646-1648

Mandel, T.E. and Kennedy, M. (1978). The differentiation of murine thymocytes in vivo and in vitro. Immunology 35 : 317-331

Mandel, T.E. and Russell, P.J. (1971). Differentiation of foetal mouse thymus. Ultrastructure of organ cultures and of subcapsular grafts. Immunology 21 : 659-674

Maroni, E.S. and De Sousa, M.A.B. (1972). The lymphoid organs during pregnancy in the mouse. A comparison between a syngeneic and an allogeneic mating. Clin. Exp. Immunol. 13 : 107-124

Maroni, E.S. and Parrott, D.M.V. (1973). Progressive increase in cell mediated immunity against paternal transplantation antigens in parous mice after multiple pregnancies. Clin. Exp. Immunol. 13 : 253-262

Mattson, R., Nilsson, B. and Lindahl-Kiessling, K. (1979). An investigation of splenic enlargement in pregnant mice. Dev. Comp. Immunol. 3 : 683-695

Metcalf, D. (1956). The origin of the plasma lymphocytosis stimulating factor. Brit. J. Cancer 10 : 442-457

Metcalf, D. (1961). The behaviour of thymus grafts in high and low leukaemia strains of mice. Aust. J. Exp. Biol. Med. Sci. 39 : 441-449

Metcalf, D. (1964). The thymus and lymphopoiesis. in, The thymus in immunobiology (Eds. Good, R.A. and Gabrielson, A.E.) Hoeber, N.Y., pp. 150-182

Metcalf, D. and Ishidate, M. (1961). PAS positive giant cells in the mouse thymus cortex. Nature 191 : 305

Metcalf, D. and Moore, M.A.S. (1971). Haemopoietic Cells. North Holland Publishing Co., Amsterdam

Metcalf, D. and Wiadrowski, M. (1966). Autoradiographic analysis of lymphocyte proliferation in the thymus and in thymic lymphoma tissue. Cancer Res. 26 : 483-491

Michalke, W.D. (1969). Thymic lymphopoiesis and cell loss in new born mice. Blood 33 : 541-544

Micklem, H.S., Ogden, D.A. and Pritchard, H. (1972). Influence of cutaneous sensitisation with oxazolone on recruitment of myelogenous stem cells in the thymus. Clin. Exp. Immunol. 12 : 103-110

Miller, J.F.A.P. and Osoba, D. (1963). The role of the thymus in the origin of immunological competence. in, The immunologically competent cell. (Eds. Wolstenholme, G.E.W. and Knight, J.) Little Brown and Co. Boston, pp. 62-76

Moore, M.A.S. and Metcalf, D. (1970). Ontogeny of the haemopoietic system : Yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br. J. Haemat. 18 : 279-297

Moore, M.A.S. and Owen, J.J.T. (1967a). Chromosome marker studies in the irradiated chick embryo. Nature 215 : 1081-1082

Moore, M.A.S. and Owen, J.J.T. (1967b). Experimental studies on the development of the thymus. J. Exp. Med. 126 : 715-726

Moore, M.A.S. and Owen, J.J.T. (1971) in, Metcalf and Moore (1971)

Mosier, D.E. and Cantor, H. (1971). Functional maturation of mouse lymphocytes. Eur. J. Immunol. 1 : 459-461

Mosier, D.E. and Pierce, C.W. (1972). Functional maturation of thymic lymphocyte populations in vitro. J. Exp. Med. 136 : 1484-1500

Murgita, R.A , Goidl, E.A., Kontiainen, S. and Wigzell, H. (1977). Alfa-fetoprotein induces T suppressor cells in vitro. Nature 267 : 257-259

McEndy, D.P., Boon, M.C. and Furth, M.D. (1944). On the role of thymus, spleen and gonads in the development of leukaemia in high leukaemia stock of mice. Cancer Res. 4 : 377-383

Nakamura, K. and Metcalf, D. (1961). Quantitative cytological studies on thymic lymphoid cells in normal, pre-leukaemic and leukaemic mice. Brit. J. Cancer 15 : 306-315

Olding, L.B. and Oldstone, M.B.A. (1974). Lymphocytes from human newborns abrogate mitosis of their mother's lymphocytes. Nature 249 : 161-162

Olsson, L. and Claesson, M.H. (1975). Studies on the regulation of lymphocyte production in the murine thymus and some effects of a crude thymus extract. Cell Tissue Kinet. 8 : 491-502

Oosterom, R., Kater, L. and Oosterom, J. (1979). Effects of human thymic epithelial-conditioned medium on mitogen responsiveness of human and mouse lymphocytes. Clin. Immunol. Immunopath. 12 : 460-470

Opelz, G. and Terasaki, P. (1974). Lymphocyte antigenicity loss with retention of responsiveness. Science 184 : 464-466

Osmond, D.G. and Nossall, G.J.V. (1974a). Differentiation of lymphocytes in mouse bone marrow (1). Quantitative radio-autographic studies of antiglobulin binding by lymphocytes in bone marrow and lymphoid tissues. Cell. Immunol. 13 : 117-131

Osmond, D G. and Nossall, G.J.V. (1974b). Differentiation of lymphocytes in mouse bone marrow (2). Kinetics of maturation and renewal of antiglobulin binding cells studied by double labelling. Cell. Immunol. 13 : 132-145

Osmond, D.G. and Yoshida, Y. (1970). Blastogenic transformation in lymphocyte rich fractions of guinea pig and rat bone marrow. in, 4th Ann. Leucocyte culture conference, Appleton-Century-Crofts, N.Y., pp. 97-109

Osoba, D. (1965). Immune reactivity in mice thymectomised soon after birth : Normal response after pregnancy. Science 147 : 298-299

Osoba, D. (1973). Effect of pregnancy on the restoration of immunological responses in neonatally thymectomised female mice. in, Contemporary topics in immunobiology. Vol. 2: Thymus dependency. (Eds. Davies, A.J.S. and Carter, R.L.) Plenum, N.Y., pp. 293-297

Osoba, D. and Miller, J.F.A.P. (1963). Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. Nature 199 : 653-654

Osoba, D. and Miller, J.F.A.P. (1964). The lymphoid tissues and immune responses of neonatally thymectomised mice bearing thymic tissue in millipore diffusion chambers. J. Exp. Med. 119 : 177-193

Owen, J.J.T. and Raff, M.C. (1970). Studies on the differentiation of thymus-derived lymphocytes. J. Exp. Med. 132 : 1216-1232

Owen, J.J.T. and Ritter, M.A. (1969). Tissue interactions in the development of thymus lymphocytes. J. Exp. Med. 129 : 431-437

Owen, J.J.T., Cooper, M.D. and Raff, M.C. (1974). In vitro generation of B lymphocytes in mouse foetal liver, a mammalian bursa equivalent. Nature 249 : 361-363

Owen, J.J.T., Jordan, R.K. and Raff, M.C. (1975). The development of the thymus in the nude mouse. Eur. J. Immunol. 5 : 653-655

Paetkau, V., Mills, G., Gerhart, S. and Monticone, V. (1976). Proliferation of murine thymic lymphocytes in vitro is mediated by the Con A induced release of a lymphokine (co-stimulator). J. Immunol. 117 : 1320-1324

Pantelouris, E.M. (1968). Absence of thymus in a mouse mutant. Nature 217 : 370-371

Pantelouris, E.M. (1971). Observations on the immunobiology of "nude" mice. Immunology 20 : 247-252

Papiernik, M., Nabarra, B. and Bach, J.F. (1975). In vitro culture of functional human thymic epithelium. Clin. Exp. Immunol. 19 : 281-287

Pepper, F.J. (1961). The effect of age, pregnancy and lactation on the thymus gland and lymph nodes of the mouse. J. Endocr. 22 : 335-348

Persike, E.C. (1940). Involution of thymus during pregnancy in young mice. Proc. Soc. Exp. Biol. Med. 45 : 315-317

Phillips, R.A. and Miller, R.G. (1970). Physical separation of haemopoietic stem cells from cells causing graft versus host disease (1). Sedimentation properties. J. Immunol. 105 : 1168-1174

Pierce, C.W. and Kapp, J.A. (1978). Functions of macrophages in antibody responses in vitro. Fed. Proc. 37 : 86-90

Pierpaoli, W. and Besedovsky, H.O. (1975). Failure of thymus factor to restore transplantation immunity in athymic mice. Br. J. Exp. Path. 56 : 180-182

Prchal, J.T., Throckmorton, D.W., Carroll, A.J., Fuson, E.W., Gams, R.A. and Prchal, J.F. (1978). A common progenitor for human myeloid and lymphoid cells. Nature 274 : 590-591

Press, O.W. and Rosse, C. (1977a). Qualitative differences in the lymphocyte PHA response between the bone marrow and other lymphoid organs. Cell. Immunol. 28 : 206-217

Press, O.W. and Rosse, C. (1977b). Thymic independence of the bone marrow lymphocyte PHA response demonstrated in nude mice. Cell. Immunol. 28 : 218-225

Press, O.W. and Rosse, C. (1978). Differences in lifespan and rate of turnover between PHA responsive cells of the bone marrow and of peripheral lymphoid organs. Immunology 34 : 539-547

Press, O.W., Rosse, C. and Clagett, J. (1977). PHA-induced differentiation and blastogenesis of precursor T cells from mouse bone marrow. J. Exp. Med. 146 : 735-746

Pritchard, H. and Micklem, H.S. (1973). Haemopoietic stem cells and progenitors of functional T lymphocytes in the bone marrow of nude mice. Clin. Exp. Immunol. 14 : 597-607

Radov, L.A., Sussdorf, D.H. and McCann, R.L. (1975). Relationship between age of allogeneic thymus donor and and immunological restoration of athymic (nude) mice. Immunology 29 : 977-988

Raff, M.C. (1969). Theta isoantigen as a marker of thymus derived lymphocytes in mice. Nature 224 : 378-379

Raff, M.C. (1971). Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transplant. Rev. 6 : 52-80

Raviola, E. and Karnovsky, M.J. (1972). Evidence for a blood-thymus barrier using electron-opaque tracers. J. Exp. Med. 136 : 466-498

Reif, A.E. and Allen, J.M.V. (1964). The AKR thymic antigen and its distribution in leukaemias and nervous tissue. J. Exp. Med. 120 : 413-433

Riches, A.C., Thomas, D.B., Briscoe, C.V., Sharp, J.G. and Littlewood, V. (1976). The morphological equivalent of the hematopoietic stem cell compartment II. Kinetic studies. Wadley Medical Bulletin 6 : 35-48

Ritter, M.A. (1978). Embryonic mouse thymus development: Stem cell entry and differentiation. Immunology 34 : 69-75

Ritter, M.A. and Morris, R.J. (1980). Thy 1 antigen: Selective association in lymphoid organs with vascular basement membrane involved in lymphocyte recirculation. Immunology 39 : 85-91

Robinson, J.H. and Owen, J.J.T. (1976). Generation of T cell function in organ culture of foetal mouse thymus I. Mitogen responsiveness. Clin. Exp. Immunol. 23 : 347-354

Robinson, J.H. and Owen, J.J.T. (1977). Generation of T cell function in organ culture of foetal mouse thymus II. MLC reactivity. Clin. Exp. Immunol. 27 : 322-327

Robinson, J.H. and Owen, J.J.T. (1978). Transplantation tolerance induced in foetal mouse thymus in vitro. Nature 271 : 758-760

Rocklin, R.E., Zuckerman, J.E., Alpert, E. and David, J.R. (1973). Effect of multiparity on human maternal hypersensitivity to foetal antigen. Nature 241 : 130-131

Roelants, G.E. (1976). Ontogeny of T lymphocytes studied in athymic and foetal mice. in, Immune reactivity of lymphocytes. (Eds. Feldman, M. and Globerson, A.) Plenum Press, N.Y. pp. 59-62

Ropke, C. (1977a). Theta-bearing cells in the bone marrow of thymus deprived mice. Scand. J. Immunol. 6 : 227-234

Ropke, C. (1977b). Kinetics of theta-positive and theta-negative lymphocytes in thymus-deprived and normal mice: an autoradiographic study. Scand J. Immunol. 6 : 291-297

Rosenstreich, D.L. and Mizel, S.B. (1978). The participation of macrophages and macrophage cell lines in the activation of T lymphocytes by mitogens. Immunological Rev. 40 : 102-135

Rosenthal, A.S., Bareinski, M.A. and Rosenwasser, L.J. (1978). Functions of macrophages in genetic control of immune responsiveness. Fed. Proc. 37 : 79-85

Rosse, C. (1976). Small lymphocytes and transitional cell populations of the bone marrow. Their role in the mediation of immune and haemopoietic progenitor cell functions. Int. Rev. Cytol. 45 : 156-290

Rosse, C. and Press, O. (1978). The differentiation of B and T lymphocytes from precursor cells resident in the bone marrow. Blood Cells 4 : 65-85

Rotter, V., Globerson, A., Nakamura, I. and Trainin, N. (1973). Studies on characterisation of the lymphoid target cell for activity of a thymus humoral factor. J. Exp. Med. 138 : 130-142

Rouse, R.V., Van Ewijk, W., Jones, P.P. and Weissman, I. (1979). Expression of MHC antigens by mouse thymic dendritic cells. J. Immunol. 122 : 2508-2515

Ruppert, B.L. and Richie, E.R. (1977). PHA response of murine spleen cells during pregnancy and inhibition of normal PHA response by pregnancy or post-partum serum. Exp. Haemat. 5 : 59-68

Saint-Marie, G. and Leblond, C.P. (1958). Tentative pattern for renewal of lymphocytes in cortex of rat thymus. Proc. Soc. Exp. Biol. Med. 97 : 263-270

Saint-Marie, G. and Leblond, C.P. (1964). Thymus cell population dynamics. in, The thymus in immunobiology (Eds. Good, R.A. and Gabrielson, A.E.) Hoeber, N.Y., pp. 207-235

Santisteban, G.A. and Dougherty, T.F. (1954). Comparison of the influence of adrenocortical hormones on the growth and involution of lymphatic organs. Endocrinology 54 : 130-146

Sato, V.L., Waksal, S.D. and Herzenberg, L.A. (1976). Identification and separation of pre-T cells from nu/nu mice. Differentiation by preculture with thymic and reticuloepithelial cells. Cell. Immunol. 24 : 173-185

Scheid, M.P. (1973). Differentiation of T cells induced by preparations from thymus and by non-thymic agents. J. Exp. Med. 138 : 1027-1032

Scheid, M.P., Goldstein, G., Hammerling, U. and Boyse, E.A. (1975). Lymphocyte differentiation from precursor cells in vitro. Ann. N.Y. Acad. Sci. 249 : 531-540

Schlesinger, D.H., Goldstein, G., Scheid, M.P. and Boyse, E.A. (1975). Chemical synthesis of a peptide fragment of thymoprotein II that induces selective T cell differentiation. Cell. 5 : 367-370

Schlesinger, M. (1972). Antigens of the thymus. Progr. Allergy 16 : 214-299

Schlesinger, M. and Golokai, V.K. (1967). Loss of thymus-distinctive serological characteristics in mice under certain conditions. Science 155 : 1115-1116

Schonenberg, M.D. and Mark, R. (1964). Cytoplasmic interaction between macrophages and lymphocytic cells in antibody synthesis. Science 143 : 964-966

Schulte-Wisserman, H., Manning, D. and Hong, R. (1978). Transplantation of cultured thymic fragments I. Morphologic and technical considerations. Scand J. Immunol. 8 : 387-397

Scollay, R.G., Kochen, M., Butcher, E. and Weissman, I.L. (1978). Lyt markers on thymus cell migrants. Nature 276 : 79-80

Scollay, R. and Weissman, I.L. (1980). T cell maturation: Thymocytes and thymus migrant subpopulations defined with monoclonal antibodies to the antigens Lyt-1, Lyt-2 and ThB. J. Immunol. 124 : 2841-2844

Scollay, R.G., Butcher, E.C. and Weissman, I.L. (1980). Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. Eur. J. Immunol. 10 : 210-218

Scothorne, R.J. and McGregor, I.A. (1955). Cellular changes in lymph nodes and spleen following skin homografting in the rabbit. J. Anat. 89 : 283-292

Sharp, J.A. (1966). Adhesion of lymphocytes to large motile cells in cultures of rabbit thymus. Nature 209 : 828-829

Sharp, J.A. (1971). The association of lymphocytes with larger motile cells in cultures of mammalian thymus. J. Pathol. 103 : 87-95

Sharp, J.G. and Thomas, D.B. (1971). The effects of mustine hydrochloride on the colony forming units of murine bone marrow. Acta Haematologica 46 : 271-274

Shaw, J., Caplan, B., Paetkau, U., Pilarski, L.M., Delovitch, T.L. and McKenzie, I.F.C. (1980). Cellular origins of co-stimulator Interleukin 2 and its activity in cytotoxic T lymphocyte responses. J. Immunol. 124 : 2231-2239

Shortman, K. and Jackson, H. (1974). The differentiation of T lymphocytes I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. Cell. Immunol. 12 : 230-246

Sigurdsson, B. (1942). A new method for demonstrating cyto-antibodies in vitro. Proc. Soc. Exp. Biol. Med. 50 : 62-66

Singh, U. and Owen, J.J.T. (1975). Studies on the effects of various agents on the maturation of thymus stem cells. Eur. J. Immunol. 5 : 286-288

Singh, U. and Owen, J.J.T. (1976). Studies on the maturation of thymus stem cells : The effects of catecholamines, histamine and peptide hormones on expression of T cell alloantigens. Eur. J. Immunol. 6 : 59-62

Small, M. and Trainin, N. (1967). Increase in antibody forming cells of neonatally thymectomised mice receiving calf thymus extract. Nature 216 : 377-379

Smith, C. (1965). Studies on the thymus of the mammal XIV. Histology and histochemistry of embryonic and early postnatal thymuses of C57BL/6 and AKR strain mice. Am. J. Anat. 117 : 611-223

Smith, K.A., Gillis, S. and Baker, P. (1979). The role of soluble factors in the regulation of T cell immune responsiveness. in, The molecular basis of immune cell function. (Ed. Kaplan, J.G.) Elsiever North Holland, Amsterdam, pp. 223-237

Strelkauskas, A.J., Wilson, B.S. and Dray, S. (1975). Inversion of levels of human T and B cells in early pregnancy. Nature 258 : 331-332

Stutman, O. (1975). Humoral thymic factors influencing post-thymic cells. Ann. N.Y. Acad. Sci. 249 : 89-105

Stutman, O. (1977). Two main features of T cell development: Thymic traffic and post-thymic maturation. in, Contemporary topics in immunobiology (Ed. Stutman, O.) Plenum, N.Y.

Stutman, O. (1978). Intrathymic and extrathymic T cell maturation. Immunological Rev. 42 : 138-184

Talmage, D.W. and Dart, G.A. (1978). Effect of O_2 pressure during culture on survival of mouse thyroid allografts. Science 200 : 1066-1068

Talmage, D.W., Dart, G., Radovich, J. and Lafferty, K.J. (1976). Activation of transplantation immunity: Effect of donor leukocytes on thyroid allograft rejection. Science 191 : 385-387

Taylor, R.B. (1964). Pluripotential stem cells in mouse embryo liver. Brit. J. Exp. Path 46 : 376-383

Ternynck, T. and Avrameas, S. (1976). A new method using p-benzoquinone for coupling antigens and antibodies to marker substances. Ann. Immunol. (Inst. Pasteur) 127C : 197-208

Ternynck, T. and Avrameas, S. (1977). Conjugation of p-benzoquinone treated enzymes with antibodies and Fab fragments. Immunochemistry 14 : 767-774

Till, J.E. and McCullough, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14 : 213-222

Trainin, N. and Linker-Israeli, M. (1967). Restoration of immunologic reactivity of thymectomised mice by calf thymus extracts. Cancer Res. 27 : 309-313

Trainin, N. and Small, M. (1970). Studies on some physicochemical properties of a thymus humoral factor conferring immunocompetence on lymphoid cells. J. Exp. Med. 132 : 885-890

Trainin, N., Small, M. and Globerson, A. (1969). Immunocompetence of spleen cells from neonatally thymectomised mice conferred *in vitro* by a syngeneic thymus extract. J. Exp. Med. 130 : 765-896

Trainin, N., Small, M., Zipori, D., Umiel, T., Kook, A.I. and Rotter, V. (1975). Characteristics of THF, a thymic hormone. in, The biological activity of thymic hormones. (Ed. Van Bekkum, D.W.) Kooyker Scientific Publications, Rotterdam, pp. 117-144

Trowell, O.A. (1954). A modified technique for organ culture *in vitro*. Exp. Cell. Res. 6 : 246-248

Trowell, O.A. (1958). The lymphocyte. Int. Rev. Cytol. 7 : 236-293

Tufveson, G. (1976). Role of the thymus in generation of lymphocyte functions. I. Demonstration of lymphocytes reactive to mitogens and allogeneic cells in the embryonic mouse thymus in organ culture. Scand. J. Immunol. 5 : 901-907

Turner, M.S. Hurst, J.M. and Yoffey, J.M. (1967). Studies on hypoxia VII. Effect of hypoxia and post-hypoxic polycythaemia (rebound) on mouse marrow and spleen. Brit. J. Haemat. 13 : 942-948

Tyndall, R.L., Otten, J.A., Proffitt, M.R., Bowles, N.D. and Tennant, R.W. (1972). Some similarities in the responses of mice to pregnancy and leukemogenesis. Int. J. Cancer, 9 : 584-594

Ulrich, F. (1979). Protease potentiation of thymocyte blastogenesis. Immunology 38 : 705-715

Unanue, E.R., Beller, D.I., Calderon, J., Kiely, J.M. and Stadecker, M.J. (1976). Regulation of immunity and inflammation by mediators from macrophages. Am. J. Pathol. 85 : 465-478

Van den Engh, G., Russell, and De Cicco, D. (1978). Surface antigens of haemopoietic stem cells: The expression of BAS, Thy 1 and H.2 antigens on CFU-S. Exp. Haemat. Today, pp. 9-15

Van den Tweel, J.G. and Walker, W.S. (1977). Macrophage induced thymic lymphocyte maturation. Immunology 33 : 817-825

Verdun, P. (1898) as quoted by Metcalf and Moore (1971)

Waksal, S.D., Cohen, I.R., Waksal, H.W., Wekerle, H., St.Aere, R.L. and Feldman, M. (1975). Induction of T cell differentiation in vitro by thymic epithelial cells. Ann. N.Y. Acad. Sci 249 : 492-498

Waksal, S.D., Smolinsky, S., Cohen, I.R. and Feldman, M. (1976). Transformation of thymocytes by thymic epithelium derived from AKR mice. Nature 263 : 512-514

Waksman, B.H. and Namba, Y. (1976). On soluble mediators of immunologic regulation. Cell. Immunol. 21 : 161-176

Warra, D.W. and Ammann, A.J. (1975). Activation of T cell rosettes in human immunodeficient patients by thymosin. Ann. N.Y. Acad. Sci. 249 : 308-315

Watkins, E.B. and Sharp, J.G. (1979). Growth and survival of irradiated, cultured mouse thymic non-lymphoid cells. J. Reticuloendothel. Soc. 26 : 209-219

Watson, J., Gillis, S., Marbrook, J., Mochizuki, D. and Smith, K. (1979). Biochemical and biological characterisation of lymphocyte regulatory molecules 1. Purification of a class of murine lymphokines. J. Exp. Med. 150 : 849-861

Weiss, L. (1977). The blood cells and haemopoietic tissues. McGraw-Hill, USA, Chapter 13.

Weissman, I.L. (1973). Thymus cell maturation: Studies on the origin of cortisone resistant thymic lymphocytes. J. Exp. Med. 137 : 504-510

Wekerle, H. and Ketelsen, U-P. (1980). Thymic nurse cells - Ia bearing epithelium involved in T lymphocyte differentiation. Nature 283 : 402-404

Wekerle, H., Feldmann, M. and Cohen, I.R. (1973). Thymus reticulum cell cultures confer T cell properties on spleen cells from thymus-deprived animals. Eur. J. Immunol. 3 : 745-748

Wekerle, H., Paterson, B., Ketelsen, U-P. and Feldman, M. (1975). Striated muscle fibres differentiate in monolayer cultures of adult thymus reticulum. Nature 256 : 493-494

Wekerle, H., Ketelsen, U-P. and Ernst, M. (1980). Thymic nurse cells. Lympho-epithelial cell complexes in murine thymuses: Morphological and seriological characterisation. J. Exp. Med. 151 : 925-944

Whang, J.E., Frei, E , Tjio, J.H., Carbone, P.P. and Brecher, G. (1963). The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukaemia. Blood 22 : 664-673

Willis-Carr, J.I., Ochs, H.D. and Wedgwood, R.J. (1978). Induction of T lymphocyte differentiation by thymic epithelial cell monolayers. Clin. Immunol. Immunopath. 10 : 315-324

Wiseman, L.L. (1977). Contact inhibition and the movement of metal, glass and plastic beads within solid tissues. Experientia 33 : 734-735

Woody, J.N. (1972). Effect of calf thymus extracts on mouse bone marrow cells. in, 7th Leukocyte Cult. Conf. (Ed. Daguillard, F.) Appleton-Century-Crofts N.Y., p. 513

Wortis, H.H. (1971a). Immunological responses of nude mice. Clin. Exp. Immunol. 8 : 305-307

Wortis, H.H., Nehlsen, S. and Owen, J.J.T. (1971b). Abnormal development of the thymus in nude mice. J. Exp. Med. 134 : 681-692

Wu, A.M., Till, J.E., Siminovitch, L. and McCullough, E.A. (1968). Cytological evidence for a relationship between normal haematopoietic colony forming cells and cells of the lymphoid system. J. Exp. Med. 127 : 455-463

Yang, W.C. and Osmond, D.G. (1979). Maturation of bone marrow lymphocytes I. Quantitative rosetting methods of detecting Fe and C' receptors and surface Ig. J. Immunol. Methods 25 : 211-225

Yoffey, J.M. (1966). Bone marrow reactions. Edward Arnold, London, p. 115

Yoffey, J.M., Patinkin, D. and Grover, N.B. (1978). The short production pathway for marrow lymphocytes: Significance of the size spectrum of the lymphocyte-transitional cell compartment. Israel J. Med. Sci. 14 : 1247-1251

Zaitoun, A.M., Lauder, I. and Aherne, W.A. (1979). Cell population kinetic profile of the mouse thymus and the changes induced by prednisolone. Cell Tissue Kinet. 12 : 191-201

Zinkernagel, R.M. and Doherty, P. (1979). MHC restricted cytotoxic T cells: Studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity, function and responsiveness. Adv. Immunol. 27 : 51-177

Zisblatt, M., Goldstein, A.L., Lilly, F. and White, A. (1970). Acceleration by thymosin of the development of resistance to murine sarcoma virus induced tumour in mice. P.N.A.S. 66 : 1170-1174

Zuckerman, S.H., Ackerman, S.K. and Douglas, S.D. (1979). Long-term peripheral blood monocyte cultures: establishment, metabolism and morphology of primary human monocyte-macrophage cell cultures. Immunology 38 : 401-411